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AN INVESTIGATION OF THE DCCD INHIBITION
OF MITOCHONDRIAL ATPase

A Thesis submitted in fulfilment of the requirements for
the degree of Doctor of Philosophy of the University of
Warwick.

by

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Summary

The mechanism of DCCD inhibition of ATP synthetase, and the components of the mitochondrial membrane with which DCCD interacts have been investigated. It has been shown that DCCD inhibits ATP-dependant reactions in rat liver mitochondria and that ^{14}C -DCCD is covalently bound to a proteolipid with a molecular weight of 10,000 daltons. This proteolipid may be synthesised on mitoribosomes. The role of the membrane in the mechanism of inhibition of the Mg^{2+} ATPase has been demonstrated by perturbation of the membrane with diethyl ether, such that inhibitor sensitivity, but not enzymic activity, is destroyed.

A series of oligomycin resistant mutants of Saccharomyces cerevisiae have been found to be cross-resistant to DCCD. An oligomycin (DCCD) sensitive ATPase has been prepared from the mitochondria of these mutants, and the mutant enzyme shown to possess a lowered sensitivity to DCCD. It is suggested that one of four subunits of the ATPase is the site of action of DCCD. It has been found that the smallest subunit will bind ^{14}C DCCD when the mitochondrial membrane is depleted of F_1 and OSCP. This subunit has been extensively purified from mitochondria of both parental and oligomycin (DCCD) resistant strains of S. cerevisiae and the mutant peptide has been shown to differ in composition from that derived from the parental strain.

Abbreviations

ADP	Adenosine 5' diphosphate
AMP-PNP	Adenylyl imidodiphosphate
Asi	Arsenate
ATP	Adenosine 5' triphosphate
ATPase	ATP phosphohydrolase (E.C. 3.6.1.4.)
DCCD	Dicyclohexylcarbodiimide
DCU	Dicyclohexylurea
DNP	Dinitrophenol
EDTA	Ethylene diamine tetraacetic acid
ETP _H	Sonic particles produced from beef heart mitochondria
F ₁	Soluble mitochondrial ATPase
NCCD	N-(2,2,6,6,-tetramethyl-piperidyl-1-oxyl) N'-(cyclohexyl)-carbodiimide
OS-ATPase	Oligomycin sensitive ATPase
OSCP	Oligomycin sensitivity conferring protein
Pi	Phosphate
SDS	Sodium dodecyl sulphate
SMP	Submitochondrial particles
SMP (NaBr)	Sodium bromide extracted SMP
SMP (NH ₄ OH)	Ammonia extracted SMP
SMP (Et ₂ O)	Ether extracted particles
TCA	Trichloroacetic acid
Tris	Tris-hydroxymethylaminomethane
Y _{ETP}	Sonic particles prepared from yeast mitochondria
CFo-F ₁	Oligomycin sensitive ATPase from bovine heart mitochondria.

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CHAPTER 1 INTRODUCTION

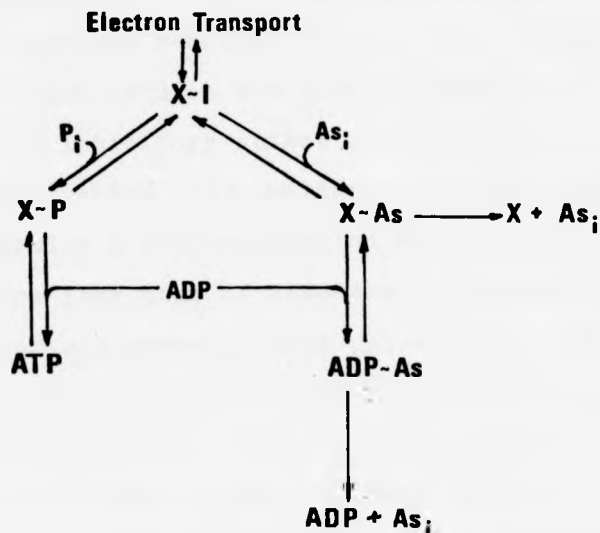
1.1. The role of Mg^{2+} ATPase in oxidative phosphorylation

The mitochondrial Adenosine 5'-triphosphatase (ATPase; ATP phosphohydrolase, E.C. 3.6.1.4.) is a complex enzyme system, naturally occurring as a part of a membrane bound complex, which catalyses the hydrolysis of Adenosine 5' triphosphate (ATP) to Adenosine 5' diphosphate (ADP) and inorganic phosphate (Pi). The first suggestion that the Mg^{2+} ATPase activity of the mitochondrion might be associated with oxidative phosphorylation was made by Lardy in 1945 (1). The mechanism by which synthesis of ATP from ADP and Pi is coupled to the oxidation of intermediary metabolites by the electron transfer system is a matter of some controversy, but proponents of the chemical hypothesis (2), the chemiosmotic hypothesis (3), and the conformational hypothesis (4, 5) all identify the terminal phosphoryl transferase with Mg^{2+} ATPase.

The chemical hypothesis was formulated by analogy to glyceraldehyde-3-phosphate oxidation. The demonstration that energy transfer can take place between the individual coupling sites indicates that coupling may proceed by way of non-phosphorylated intermediates. These are postulated to be the electron carriers in the respiratory chain. The reaction pathways leading to the formation of ATP are suggested to be

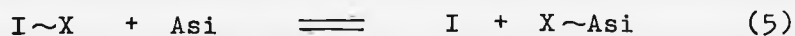


Footnote: Arsenate stimulated respiration is inhibited by phosphate and this inhibition is relieved by ADP. In the presence of aurovertin the arsenate stimulated respiration is still inhibited by phosphate but the inhibition is no longer relieved by ADP. This is consistent with equations (4) and (5), and establishes the site of aurovertin inhibition as lying between X-P and ATP. At low concentrations of aurovertin, the inhibitor will inhibit arsenate stimulated respiration, suggesting that when aurovertin is absent, X-As is hydrolysed slowly and that most of the arsenate stimulated respiration is accounted for by participation of endogenous ADP (213).





analysis of the O^{18} products following the H_2O^{18} into Pi and ATP (6, 7, 8) shows that O^{18} enters at the point of phosphate uptake, and that the oxygen in the terminal pyrophosphate linkage of ATP must be provided by a terminal oxygen from ADP rather than Pi. These observations suggest a $X \sim ADP$ intermediate in the formation of ATP as outlined in equation 4. The intermediate $I \sim X$ is postulated to explain the finding that DNP and arsenate act at two different levels of the energy transfer system, as revealed by studies with oligomycin. Oligomycin inhibits oxidative phosphorylation (9), the ATP - Pi exchange reaction (10) and the ATPase (11). Arsenate, which induces the ATPase reaction (12), does not relieve the inhibitory effect of oligomycin on tightly coupled respiration (13), unlike uncoupling agents. This phenomenon permits a differentiation of the site of action of uncouplers from that of arsenate. Furthermore, it shows that oligomycin probably acts prior to the entry of phosphate. Oligomycin probably acts by inhibiting equation 2, accounting for its inhibition of arsenate uncoupled, but not uncoupler stimulated respiration. Arsenate acts by an arsenolytic reaction as described in equations 5 and 6.



Uncouplers are thought to act at $A \sim I$.

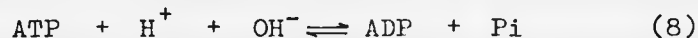
The chemiosmotic hypothesis was postulated by Mitchell (3), to provide an explanation for the requirement of an intact membrane for phosphorylation, since this requirement is not implicit in the chemical-coupling hypothesis. It

also sought to explain the failure of all attempts to identify the intermediates X and I. In its simplest form, the chemiosmotic hypothesis consists of the generation of a proton gradient across the proton impermeable inner membrane by the electron transport chain. This proton gradient then drives the synthesis of ATP from ADP and Pi by means of an anisotropic ATPase.

The proposal depends on the possibility that the ATPase may lie in the membrane, isolated from the aqueous phase on both sides, but accessible to OH^- ions on one side and to H^+ on the other. If we consider the hydrolysis of ATP,



This reaction may be written



showing that, given such an anisotropic ATPase, hydrolysis of ATP would be accompanied by the effective translocation of protons from one side of the membrane to the other. Similarly, the reverse reaction, ATP synthesis, could be effected by a flow of protons across the membrane in the opposite direction.

In the conformational hypothesis (4, 5) the driving force for the synthesis of ATP is a conformational change in the coupling apparatus. The basic unit of mitochondrial energy conservation is suggested to be a 'supermolecule' (14). The supermolecule consists of two components, one of which catalyses a polarising reaction, and one which catalyses a depolarising reaction. The depolarising element, the ATPase, is polarised by separation of electrons and protons in the electron transfer complexes. A conformational

strain is set up in the ATPase such that conditions are energetically suitable for a chemical reaction between ADP and Pi. When bond formation takes place, the conformational strain in the supermolecule is removed. Other postulated depolarising components, the transhydrogenase, transprotonase and transphosphorylase act via a similar mechanism, catalysing the transfer of hydride ion, protons, and phosphoryl groups respectively (14).

Supportive evidence for the proposed identity of Mg^{2+} ATPase and the terminal phosphoryl transferase in ATP synthesis is often ambiguous. Classically, investigation of the mechanism of oxidative phosphorylation has been carried out by preparation of respiring submitochondrial particles depleted of one or more proteins and lacking phosphorylation. Restoration of phosphorylation coupled to respiration (monitored by inhibitor sensitive ATP synthesis or energy linked reactions) may be achieved by addition of soluble proteins removed from submitochondrial particles (coupling factors). Over 20 such coupling factors of varying purity have been described (15, 16), several of which possess ATPase activity (17 - 22). The best characterised of these coupling factors is F_1 (17). Removal of F_1 from mitochondrial particles results in loss of oxidative phosphorylation and membrane bound ATPase activity (23). Addition of F_1 to depleted particles stimulates oxidative phosphorylation, ATP/Pi exchange and reversed electron transfer (17, 24, 25). Antibody to F_1 inhibits oxidative phosphorylation and the membrane bound ATPase (26). However, this stimulation by F_1 is due, at least in part, to some organising ability of

the protein on the mitochondrial membrane. F_1 , dissociated and inactivated by oxidation of sulphhydryl groups, will still stimulate oxidative phosphorylation in depleted particles (27). Further evidence for F_1 as a structural entity is provided by the work of Schatz (28). F_1 from yeast mitochondria stimulates oxidative phosphorylation in beef heart mitochondrial particles if some beef heart F_1 is present. Antibody to beef heart F_1 does not remove the ATPase activity of yeast F_1 , but abolishes the coupling activity. Antibody to yeast F_1 inhibits the ATPase but not the coupling activity (28).

Perhaps the strongest evidence for involvement of the ATPase in ATP synthesis is provided by inhibitor studies. Inhibitors of oxidative phosphorylation include oligomycin (10), rutamycin (29), aurovertin (18, 30), dicyclohexylcarbodiimide (31, 32), trialkyl tin chlorides (29, 33), venturicidin (34) and azide. These inhibitors also inhibit the membrane bound ATPase and a detergent solubilised preparation of the ATPase (29 - 34). Dio-9 (35) is a potent inhibitor of ATPase in yeast, but has little effect on the beef heart enzyme. However, only azide, aurovertin and Dio-9 inhibit the ATPase activity of F_1 . Moreover, the inhibitor protein of Pullman and Monroy (36), Adenylyl imidodiphosphate (AMP-PNP), and quercetin (37) inhibit both soluble and membrane bound ATPase activity but do not inhibit oxidative phosphorylation (36 - 38). The inhibitors of oxidative phosphorylation which do not inhibit F_1 may be shown to act at a site in the coupling mechanism prior to the entry of phosphate. The insensitivity of F_1 to these

inhibitors is thus explained by the lack of the proteins binding the inhibitors and which are presumably involved in the production of the high energy intermediate whether chemical, proton gradient, or charge separation. These proteins are however present in the detergent solubilised preparation (39).

1.2. Oligomycin insensitive ATPase

Preparations of ATPase from mitochondria may be classified into oligomycin sensitive and insensitive enzymes. Where data concerning the sensitivity to other inhibitors is available, the oligomycin sensitive ATPase preparations are inhibited by compounds inhibiting oxidative phosphorylation, and the oligomycin insensitive preparations are inhibited by aurovertin (and possibly azide and Dio-9). Numerous coupling factors with ATPase activity have been isolated but the most purified and best characterised are F_1 (17), Factor A (19), and the OS-ATPase of Tzagoloff (39, 40). F_1 has been purified to homogeneity from yeast (41, 42), rat liver (43, 44), and beef heart (17, 43) mitochondria. Most methods for purification of F_1 involve sonication of mitochondrial membranes followed by ion exchange chromatography and gel filtration of the 100,000g supernatants. Activities for the enzymes are of the order of 50 μ moles ATP hydrolysed/minute/mg protein. The preparations are water-soluble, lipid-free and cold labile. F_1 has an estimated molecular weight of 540,000 (45).

Factor A, although similar to F_1 in most respects, differs in that it has only a latent ATPase activity which is

stimulated by heat, and which is cold stable (19). The differences between F_1 and Factor A may be due to the absence of a heating step in the purification of Factor A (19). Sanadi has proposed that the ATPase activity is induced by a conformational change in the protein such that the availability of the active site to water is increased (46). This would seem to be borne out by the low ATPase activity of intact mitochondria and the high ATPase activity of damaged mitochondria (47). Pullman and Schatz have attributed the low ATPase activity of Factor A to the presence of ATPase inhibitor protein (48). Sanadi, however, claims that F_1 and Factor A contain the same amount of inhibitor protein.

Electrophoresis of F_1 under denaturing conditions shows that most preparations contain five subunits (49). F_1 prepared by the method of Horstman and Racker (50) contains a sixth subunit which may be the ATPase inhibitor protein. The two largest peptide subunits have molecular weights between 50,000 and 62,000 (41, 43, 44, 51 - 53). They comprise some 90% of the total protein as estimated by binding of stain in polyacrylamide gel electrophoresis. The third subunit has a molecular weight between 25,000 and 36,000 (41, 43, 44, 51 - 53), and is somewhat less than 10% of the total protein. The two minor subunits have molecular weights below 20,000. The stoichiometry of the peptides has been proposed to be A_3B_3CDE for both beef heart (49) and rat liver (53) F_1 . Evidence supporting the proposed subunit structure is (A) Electron micrographs show a hexameric arrangement of similar subunits (A and B) (B) A_3B_3CDE is

consistent with the dye binding of these subunits in SDS gel electrophoresis. It should be borne in mind that estimation of protein by staining intensity is not applicable to all proteins (56). One of the subunits of the oligomycin sensitive ATPase does not bind dye (57). (C) The number of cysteines and half cystines in F_1 is 12 (52). The arrangement A_3B_3CDE is consistent with 12 cysteines from the known amino acid composition.

All five of the subunits of beef heart F_1 have been isolated in pure form (52, 55), and similar amino acid analyses obtained from two laboratories (52, 55) for the four largest subunits. There is some disagreement about the size and amino acid composition of the smallest subunit. Knowles and Penefsky (52) suggest that the fifth subunit is a dimer, although this would seem improbable under the denaturing conditions used in SDS-gel electrophoresis. Knowles and Penefsky (52) have also suggested that the fifth subunit of F_1 is identical with the ATPase inhibitor protein of Pullman and Monroy (36), on the basis that the proteins have the same mobility in SDS gel electrophoresis and the same isoelectric point. It has since been shown that subunit five has a low inhibitory activity and that co-electrophoresis of subunit five and the ATPase inhibitor gave two distinct bands on electrophoresis. Moreover, the amino acid composition of inhibitor protein is known (10), and does not resemble that of subunit five according to Knowles and Penefsky (52), or Senior and Brooks (55).

1.3. Oligomycin sensitive ATPase

A variety of preparations of oligomycin sensitive ATPase from beef heart mitochondria have been published (58 - 62), of which the most purified is that of Swanljung (62). The majority of OS-ATPase preparations from beef heart have not been purified to any great extent, the main contaminants being cytochromes, flavins and lipids. Most of the information on the structure and composition of the OS-ATPase has been provided by the highly purified ATPase of Tzagoloff and Meagher (40), from yeast mitochondria. As in the case of Swanljung (61, 62), the ATPase is solubilised from the membrane by low concentrations of the non-ionic detergent Triton X-100, and is further purified by density gradient centrifugation. The purified preparation has a specific activity of 20 to 30 μ moles ATP hydrolysed/minute/mg protein, and is lipid activated. Unlike both F_1 and the OS-ATPase of Swanljung, it is cold stable. The level of phospholipid depletion is dependant on the concentration of triton used in the initial extraction.

All of the OS-ATPase preparations either contain phospholipids, or require phospholipid for maximal activity (40, 58 62). The OS-ATPase of Tzagoloff (60) from beef heart contains about 30% phospholipid and is not activated by addition of further phospholipid. The corresponding preparation from yeast may also contain up to 30% phospholipid depending on the extraction conditions, but no data on activation by phospholipid has been reported (40). Activation of other OS-ATPase preparations by phospholipid has been described (58 - 64).

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The most detailed studies of lipid activation are those of Swanljung (63), and Cunningham (64). Both investigators agree that all phospholipids are competent to stimulate ATPase activity. Maximal stimulation is provided by lysolecithin. Swanljung et al. (64) have shown that addition of phospholipid lowers the K_m (ATP) of the lipid depleted OS-ATPase from beef heart. Cunningham, however, has shown that the K_m (ATP) of CF_0-F_1 is exactly the same in unactivated and asolectin treated preparations (63).

Perhaps the most significant result of Cunningham is the finding that there is no stimulation of CF_0-F_1 by diglycerides, although addition of phospholipids, fatty acids, monoolein or SDS gave rise to an oligomycin sensitive ATPase. The stimulation of OS-ATPase by such a diverse group of lipids suggests that the oligomycin sensitivity of the ATPase is not conferred by the lipophilic activators. Moreover, the only common property of the activating lipids is their amphipathic nature, and it is possible that their ability to stimulate the ATPase is due to this property. Verkleij (65) has shown that diglycerides differ from phospholipids in their inability to form a bilayer structure. The amphipathic nature of phospholipids enables them to form lamellar leaflets with a hydrophobic interior and polar groups projecting into the aqueous phase. It is possible that lipophilic activators stimulate the ATPase by providing a bilayer structure which stimulates the mitochondrial membrane.

The role of phospholipids in the mechanism of ATP synthesis and inhibitor sensitivity is still obscure, but it has been suggested that one of the functions of lipids is in linking the oligomycin insensitive ATPase to the other subunits of the OS-ATPase conferring oligomycin sensitivity. This is consistent with the work of Toson (66) and Lee (67) showing that F_1 may be released from submitochondrial particles by incubation with excess phospholipid.

The OS-ATPase has a molecular weight of 468,000, based on sedimentation velocity, with an appropriate correction for 10% phospholipid content (40). The enzyme is separated into eight stained bands in SDS gel electrophoresis, and a ninth non-staining band which may be detected by incorporation of radiolabelled leucine during growth of the yeast cell (57). Some of these subunits may be accounted for as subunits held in common with F_1 , which may be prepared from the OS-ATPase by heating or salt extraction (68, 69). Tzagoloff has shown that subunits 1, 2, 3, 4 and 8 are common to both F_1 and OS-ATPase (40). The band with a molecular weight of 12,000 daltons has been shown to be composed of two proteins on the basis of their differential sensitivity to inhibitors of cytoplasmic and mitochondrial protein synthesis (75). A summary of the molecular weights of the beef heart and yeast oligomycin sensitive and insensitive ATPases is given in Table 1.

Negatively stained preparations of the inner mitochondrial membrane display a characteristic array of

TABLE 1.1. Peptide subunits of OS-ATPase and F₁

Preparation	Yeast OS-ATPase ¹	Yeast F ₁ ¹	Beef Heart OS-ATPase ^{2,3}	Beef Heart F ₁ ^{4,5}
			73,000	
	58,000	58,000	55,000	53,000
	54,000	54,000	52,000	50,000
Molecular	38,000	38,000	30,000	-
	31,000	31,000	29,000	25,000
Weight	29,000	-	20,000	-
	22,000	-	19,000	-
	18,500	-	12,500	12,500
	12,000	12,000	10,000	-
	7,500	-	8,000	7,500

1 J. Biol. Chem. 246, 7328

2 Biochem. Biophys. Res. Commun. 53, 1331

3 Biochemistry 7, 1596

4 FEBS Lett. 17, 327

5 Archiv. Biochem. Biophys. 140, 257

100Å diameter spheres bound to the inner surface (59). These 'knobs' may be removed by treatments (sonication, salt extraction) which remove the oligomycin insensitive ATPase from the membrane, and may be replaced by reconstitution of depleted membranes with pure F_1 (68, 69). The inner membrane spheres are bound to the membrane by a 50 x 30Å 'stalk' which has been identified with a coupling factor conferring oligomycin sensitivity (70). This oligomycin sensitivity conferring protein (OSCP) has a molecular weight of 18,000 and accounts for band 7 of the OS-ATPase SDS gel pattern (69). The protein is required for binding of F_1 to the membrane, and confers oligomycin sensitivity, although there is no evidence that it is the site of action of oligomycin, and it has been suggested (71) that the subunit should be renamed 'basic coupling factor' in respect of its extremely basic nature (70).

The remaining four bands comprise the 'membrane factor', so called since it is the most insoluble part of the complex and may only be solubilised from the membrane by detergents. All four proteins are extremely hydrophobic, and belong to the class of proteolipids (72). Although their function is not known, they are necessary for conferral of oligomycin sensitivity (69, 73), and may be involved in the binding of F_1 and OSCP into the mitochondrial membrane during assembly of the OS-ATPase complex (69, 73 - 75). The smallest subunit has been purified from the OS-ATPase, and directly from submitochondrial particles (57). Subunit 9 is soluble in

chloroform/methanol (2:1 v/v) and contains a high percentage of non-polar amino acids (57). The other three peptides of the membrane factor are extractable only in acidic chloroform/methanol and have not been purified (76). The beef heart OS-ATPase has been shown to contain a peptide covalently binding DCCD (77) and a proteolipid binding DCCD has been isolated from beef heart mitochondria by chloroform/methanol extraction (78). The molecular weight of this DCCD-binding protein is 10,000.

The membrane factor peptides are usually assumed to be present in a 1:1 stoichiometry with F_1 . The molecular weight of the OS-ATPase is about 470,000 and that of F_1 360,000. The molecular weights of the membrane factor peptides plus OSCP total 90,000 leaving a discrepancy of about 20,000 daltons. Beechey (71) has suggested that subunit 9 may be present in a 1:1 stoichiometry with the largest subunits of the oligomycin insensitive ATPase.

1.4 Biogenesis of ATPase components

Investigations in several laboratories (79, 80) have shown that mitochondria are semi-autonomous bodies containing informational DNA (79), mitoribosomes (80), and a mechanism for protein synthesis similar to, but distinct from, that carried out in the cytoplasm. One of the major differences between cytoplasmic and mitochondrial protein synthesis is the sensitivity to chloramphenicol and the lack of inhibition by cycloheximide exhibited by the latter (81). Amino acid incorporation into yeast mitochondria is also inhibited by other

antibiotics, such as erythromycin and lincomycin, which act in a similar manner to chloramphenicol (82). The majority of mitochondrial phospholipid and protein appears to be synthesised extramitochondrially, and subsequently transferred to the mitochondrion. Only about 10% of the total mitochondrial protein is synthesised by mitochondria in rat liver (85).

There are two main approaches to the study of the nature of the proteins synthesised by mitochondria. The first involves the isolation of mitochondria, and incubation of these preparations with radioactive amino acids. The preparations may be washed and fractionated by conventional methods. Secondly, studies involving the incorporation of amino acids in vivo into mitochondria have been used to detect the distribution of radiolabelled peptides in both rat liver and yeast. The selective inhibition of mitochondrial protein synthesis by chloramphenicol and microsomal protein synthesis by cycloheximide has been used extensively in these experiments. The use of isolated mitochondria has been criticised on several grounds. Firstly, the incorporation of amino acids is slower in isolated mitochondria than in vivo (83). Secondly, extreme care has to be taken to minimise any contribution arising from microsomal or bacterial contamination (84). It seems, however, that the products of mitochondrial protein synthesis are similar in both vitro and vivo (85).

It has been shown that isolated rat liver mitochondria incorporate amino acids into the inner mitochondrial

membrane only (86). Fractionation of the inner mitochondrial membrane indicates that the proteins synthesised by the mitochondria are among the most insoluble of those in the membrane. No radioactive amino acids are incorporated into the readily soluble proteins of the mitochondrion (86 - 88). Electrophoresis in SDS of submitochondrial particles prepared from rat liver mitochondria incubated with ^3H leucine and cycloheximide shows that the main product of mitochondrial protein synthesis has a molecular weight of about 40,000 daltons (89). Three peptides with molecular weights of 48,000; 28,000; 20,000 and three peptides of lower molecular weight may also be detected (89). Tzagoloff has shown that the main products of mitochondrial protein synthesis in yeast have molecular weights of 45,000, 29,000, 21,000, 12,000 and 7,800 (75). The results of Thomas and Williamson are in general agreement with these findings, with the exception of an additional peptide with a molecular weight of 33,000 (90). It was also found that the largest and smallest mitochondrially synthesised peptides selectively incorporated ^3H -2-glycerol (90). Further fractionation of the inner mitochondrial membrane has shown the presence of several of these peptides in the OS-ATPase (75).

Most of the information on the biosynthesis of the ATPase complex has come from the elegant experiments of Tzagoloff (57). Glucose repressed yeast grown in derepression medium containing chloramphenicol have a constant level of ATPase in the mitochondrial fraction but an ATPase activity, sensitive to antibody to F_1 , is to be

found in the post-ribosomal supernatant. This soluble ATPase is not found if cycloheximide is present (91). If chloramphenicol and ^{14}C leucine are included in the derepression medium, then the cytoplasmic protein synthesis products become labelled. Purification of the ATPase produced under these conditions gives a preparation with five peptide subunits which co-electrophorese with a ^3H -leucine labelled preparation of F_1 (92). Similar results are obtained if the soluble enzyme is prepared from petite mutants (57). It would seem therefore that all of the peptides of F_1 are synthesised outside the mitochondrion. The biosynthesis of the oligomycin sensitivity conferring protein has been investigated by essentially the same means as F_1 (69). The problem is complicated by the lack of any known enzymic activity of OSCP, and the protein must be assayed by its ability to stimulate the binding of F_1 to depleted membranes. The synthesis of OSCP has been found to be inhibited by cycloheximide, but not by chloramphenicol, indicating a cytoplasmic origin for this peptide (69). F_1 and OSCP do not appear to be present as a complex in the post-ribosomal supernatant.

Investigation of the origin of the membrane factor of OS-ATPase suffers from the same problems as studies on OSCP. No known enzymic activity can be ascribed to the membrane factor, nor is there a satisfactory method of purification of this portion of the complex. The increase in the membrane factor during derepression is therefore monitored by the ability of NaBr-Alkali extracted

particles to bind F_1 and confer oligomycin sensitivity. The ability of these membranes to bind F_1 increases during derepression, and is inhibited by both cycloheximide and chloramphenicol. If the cells are grown in chloramphenicol first and then in cycloheximide, then there is no inhibition of membrane factor synthesis; this is not true if the order of the inhibitors is reversed. These findings indicate mitochondrial synthesis of the membrane factor in the presence of cycloheximide (70). If 3H leucine is included in the derepression medium with cycloheximide, the mitochondrial protein is labelled. Extraction of such mitochondria with Triton to remove the OS-ATPase, and precipitation of the Triton extract with antisera to OS-ATPase, gives rise to a preparation displaying four radioactive subunits in SDS gel electrophoresis (75). Similar results are obtained if depleted particles, labelled in the presence of cycloheximide, are reconstituted with unlabelled F_1 and OSCP (70). The major product is a low molecular weight peptide which is identified with subunit 9 of the ATPase on the basis of migration within polyacrylamide gel (57, 75). The three other peptides migrate in the region of subunits 5, 6 and 8 (57, 75).

The four membrane factor peptides have been shown to be proteolipids, and extraction with chloroform methanol of submitochondrial particles or OS-ATPase derived from cells grown in the presence of 3H leucine and cycloheximide removes a single peptide of molecular weight 7,500 which co-electrophoreses with subunit 9 (57). The other

membrane factor subunits have not been purified but may be extracted with acidic chloroform-methanol (2:1 v/v) (57,76). Thomas and Williamson have also reported extraction of two mitochondrially synthesised peptides from yeast mitochondria by chloroform-methanol (93). These two peptides, which incorporate ^3H -glycerol have molecular weights of 11,000 and 48,000 and probably correspond to subunit 9 and a polymer or association product of subunit 9. Tzagoloff has shown that counts associated with a peptide of molecular weight 45,000 are to be found co-migrating with subunit 9 after treatment with base (57, 76).

Although there is no satisfactory preparation for OS-ATPase from rat liver mitochondria, Burke and Beattie (94) have shown that about 15% of the ^3H leucine incorporated into isolated rat liver mitochondria are extracted by chloroform-methanol. The incorporation is inhibited by chloramphenicol and carbomycin, both specific inhibitors of mitochondrial protein synthesis. Chloroform methanol extraction of labelled particles leads to a decrease in the label associated with a peptide of molecular weight 40,000, and total removal of two low molecular weight products (95). About 20% of the total label may be extracted into acidic chloroform-methanol. The results of Kuzela et al. (96) and Kadenbach (97) from in vivo incorporation into rat liver, are in general agreement with these findings. Kuzela finds that the major product is a peptide soluble in neutral chloroform methanol with a molecular weight of 9,000 (96). Burke

SYNTHESIS OF SUBUNITS OF YEAST OS-ATPase.

COMPONENT	MOLECULAR WEIGHT.	SITE OF SYNTHESIS.
F ₁	58,500	Cytoplasmic.
F ₁	54,000	Cytoplasmic.
F ₁	38,500	Cytoplasmic.
F ₁	31,000	Cytoplasmic.
F ₁	12,000	Cytoplasmic.
OSCP	18,500	Cytoplasmic.
F ₀	29,000	Mitochondrial.
F ₀	22,000	Mitochondrial.
F ₀	12,000	Mitochondrial.
F ₀	7,000	Mitochondrial.

N.B. The site of synthesis of mitochondrial components is not proven unambiguously by inhibition by either chloramphenicol or cycloheximide, due to the interdependence of the cytoplasmic and mitochondrial protein synthesising systems. The following criteria have to be met before assigning mitochondrial synthesis.

- a) Synthesis is inhibited by chloramphenicol and not by cycloheximide.
- b) The product is absent in petite mutants.
- c) Synthesis is possible in isolated mitochondria.

Conversely, the protein may be said to be nuclear coded if

- a) Synthesis is inhibited by cycloheximide but not by chloramphenicol.
- b) The protein is present in petite mutants.
- c) Synthesis is not possible in isolated mitochondria.

and Beattie have reported that chloroform-methanol extraction of lyophilised, labelled particles removes label associated with two broad bands in SDS gels in the molecular weight region 10 - 14,000. No significant counts are found in the higher molecular weight regions of the gel (95). It is possible that the 40,000 molecular weight product is produced by association with other products in the same way as the 45,000 molecular weight product in yeast.

1.5 The use of mutants in bioenergetics

The use of mutants in the elucidation of metabolic pathways has become a standard tool of the research biochemist. The mutant approach in bioenergetics has been severely limited by the lack of suitable mutants and difficulties in fractionating the membrane bound enzymes involved.

Bacterial mutants deficient in energy conservation have been produced only recently, although the genetics of Escherichia coli are well characterised, and the problems arising from synthesis due to mitochondrial DNA and nuclear DNA are not found in procaryotes. Mitochondrial and bacterial oxidative phosphorylation probably operate by similar mechanisms (98). Suitable mutants of E. coli produced by Cox and Gibson (99, 100) have defects in the ATPase, or the coupling membrane proteins, and may prove valuable in the investigation of the coupling process. Mutants of Streptococcus faecalis resistant to DCCD have been isolated and the involvement of a membrane bound

coupling factor shown (101). Suitable preparations of bacterial membrane factors have not yet been described.

Mutants derived from eucaryotic organisms, especially yeast, have provided most of our information concerning the biosynthesis and nature of the coupling system. Yeast is a particularly suitable organism for a combined genetic and biochemical investigation on several counts. Yeast is one of the simplest of eucaryotes, and may be phenotypically manipulated by defined changes in the growth conditions. Petite positive yeast strains may survive drastic alteration in their mitochondrial DNA, including total deletion. The biochemistry of yeasts is well known.

The production or isolation of suitable mutants should be followed by screening procedures to determine the method of inheritance. The isolation and screening procedures for mutants of S. cerevisiae have been reviewed (102). Following preliminary screening, the mutants should be investigated at both mitochondrial and enzyme level, the ultimate aim being to equate a point mutation with an amino acid change in a specific peptide.

The petite mutation (103) represents a considerable deletion of mitochondrial DNA and is expressed as an inability to carry out mitochondrial protein synthesis. Any property or peptide absent in petite mitochondria may therefore be assumed to be synthesised on mitoribosomes or be linked to products of such synthesis. Petites, although possessing an ATPase, do not show oligomycin sensitivity of this activity. In addition, it has been

22

shown that petites do not possess an ATP/Pi exchange. These observations may indicate that the subunits of the OS-ATPase which are responsible for conferring oligomycin sensitivity are required for effective functioning of the ATP synthetase in energy conservation.

It has also been shown that the mitochondrial membrane in petites is rather impermeable to protons, although this may be abolished by uncouplers (104). Although the membrane is capable of charge separation, it is thought that the mechanism by which charge separation arises is inoperative. This may be linked to the absence of the membrane factor peptides of the ATPase, and to a disturbance of the respiratory chain (cytochromes a and b are absent in petites). Similarly it has been reported that the atractyloside sensitive adenine nucleotide translocase is not present in petites, although this is a matter of some dispute (105, 106).

Kovac has isolated a slow growing mutant with normal respiration and cytochromes (107). This op_1 mutant has since been shown to be nuclear and has a defective adenine nucleotide translocase, but a normal ATPase (108). Other nuclear mutants of S. cerevisiae have been shown to lack subunits of F_1 (109).

An alternative approach to the isolation of mutants with altered components of the energy conservation system has been adopted by Griffiths (110 - 115). Mutants have been selected for growth on non-fermentable substrate in the presence of inhibitors of oxidative phosphorylation. Any such mutants not due to cell or mitochondrial membrane

impermeability or detoxification may be due to a modified ATPase complex. Mutants resistant to oligomycin (110, 111), triethyl tin (112, 113) and venturicidin (114, 115) have been isolated. The mutants may be grouped into Class 2 mutants, which are specifically resistant to inhibitors of oxidative phosphorylation, and Class 1 mutants, which are cross resistant to a wide range of inhibitors, uncouplers and inhibitors of protein synthesis. Class 1 mutants are considered to be general membrane mutants.

Class 2 oligomycin resistant mutants have been shown to be cytoplasmic (110) and map at two loci, OL1 and OL11, on the mitochondrial genome (111). Decreased sensitivity to oligomycin has been shown in the mitochondrial ATPase, ATP/Pi exchange and ADP stimulated respiration of the mutants, and in purified preparations of the oligomycin sensitive ATPase (116). Class 2 triethyl tin resistant mutants have also been shown to be cytoplasmic and map at the T locus (113). Decreased sensitivity to triethyl tin is found in the ATPase and ADP stimulated respiration of Class 2 mutants but is not as marked as in the oligomycin resistant mutants (113). Mitochondrial venturicidin resistant mutants (114) map at two loci, those exhibiting cross-resistance to oligomycin mapping at the OL 111 locus which is closely linked to OL 1 (114), and those exhibiting cross-resistance to triethyl tin mapping at the V 1 locus (114). It is likely from the combined biochemical and genetic data, that OL 1 and OL 111 are on the same cistron although some differences

in the response of the ATPase to oligomycin are found (114). Venturicidin mutants mapping at V 1 are very similar to the triethyl tin mutants mapping at T 1, and it seems likely that T 1 and V 1 are identical, especially since the triethyl tin mutants have since been shown to be cross-resistant to venturicidin. All of the available evidence suggests that OL 1, OL 11 and V 1 are located on separate cistrons (114).

The resistance phenomenon may be explained by a binding of oligomycin at three attachment points, modifications of which arise by mutation at loci OL 1, OL 11 and OL 111. Venturicidin binding is at two attachment points modified by OL 111 and V 1. The similarity in structure between oligomycin and venturicidin would suggest they have a common binding site (OL 111). It is also possible that OL 1, OL 11 and OL 111 represent modification of peptides not directly involved in the binding of oligomycin, but which induce an unfavourable conformation at the binding site. The binding of inhibitors to the OS-ATPase has been shown to be highly sensitive to conformational change (117).

1.6 Reactions catalysed by the OS-ATPase complex

It is difficult to see why the OS-ATPase is such a large, assymetric complex. The only known activity of the complex is the hydrolysis/synthesis of ATP, coupled to a transmembrane movement of protons (118). It has been suggested that there are two catalytic sites on the F_1 enzyme, one specialised for ATP synthesis and one

specialised for ATP utilising reaction. Support for this theory comes from the differential effects of aurovertin (18), AMP-PNP (119), quercitin (37) and the inhibitor protein of Pullman and Monroy (36) on oxidative phosphorylation and ATP requiring reactions.

It is well known that a tenfold greater concentration of aurovertin is required to inhibit the ATPase than oxidative phosphorylation. Mitchell has suggested on kinetic grounds (120) that aurovertin increases the affinity of the enzyme for ATP and decreases the affinity for ADP. Chang and Penefsky (121) have suggested that there are two aurovertin binding sites, one involved in the inhibition of oxidative phosphorylation and one involved in the inhibition of ATPase. Van de Stadt (122) has shown that aurovertin may be used as a probe of ATPase conformation, and proposes that a high affinity site, inhibiting oxidative phosphorylation, is induced by ATP and Pi, while an ADP stabilised low affinity site is responsible for the inhibition of ATPase.

The inhibitor protein of Pullman and Monroy (36), quercitin (37) and AMP-PNP do not inhibit oxidative phosphorylation, but inhibit ATPase and ATP dependant reactions. Penefsky has suggested that the site of ATP synthesis is insensitive to AMP-PNP, while a second site specialised for ATP utilising reactions is inhibited (123).

Two binding sites for ADP have been found in beef heart F_1 (124), a high affinity site (K_d 0.28 μ M) and a low affinity site (K_d 47 μ M) suggested to be the ATPase site because of the similarity of the dissociation constant

with the inhibition constant of ADP for ATPase (124).

According to Mitchell (125, 126) the membrane factor peptides conferring oligomycin and DCCD sensitivity on F_1 may also be responsible for the translocation of protons. This suggestion is supported by the experiments of Hinkle (127) showing that oligomycin and DCCD inhibit the permeability of F_1 -depleted particles to protons. Also certain bacterial mutants or bacterial vesicle preparations lacking energy linked reactions may have some degree of restoration conferred by low concentrations of DCCD or oligomycin (128). Moreover petites (103), lacking the membrane factor peptides (57, 129), have a relatively low permeability to protons, which may be increased by uncouplers (104). According to Mitchell, the membrane factor peptides may provide a translocation pathway the membrane permitting access of protons and water to F_1 . This theory is attractive in that F_1 has the same enzymic function in the membrane bound or isolated state (125).

It is possible that some of the subunits of the OS-ATPase catalyse other enzymic activities. If the membrane subunits are responsible for H^+ translocation, it may be that other transport proteins may be located in the complex. Kadenbach (131, 132) has found that chloroform-soluble proteins from rat liver mitochondria bind phosphate strongly, and that this binding is sensitive to thiol reagents such as N-ethylmaleimide which are inhibitors of phosphate transport (131). Oligomycin DCCD and tributyl tin, which inhibit ATPase activity, have no effect

on the phosphate transporter (133). It is also interesting that a proteolipid from pig heart mitochondria has been shown to possess a high affinity for glutamate (134).

It has also been suggested that at least one of the subunits of OS-ATPase may be held in common with ADP translocase (135). Triethyl tin mutants of S. cerevisiae (112) have been shown to possess cross-resistance to bongkreikic acid, a good inhibitor of ADP translocation (136, 137).

The possibility of an interaction between the ADP carrier and the coupling system of oxidative phosphorylation has been reviewed by Vignais et al (211) and receives strong support from observations showing that the phosphorylation of external ADP can be accomplished without the obligatory flux of adenine nucleotides into and out of the mitochondrial matrix (212). Vignais suggests that ATP is released as free nucleotide from the F₁-ATPase into a microaqueous space to which the ADP carrier has direct access (211).

Footnote; summation of the effects of oligomycin and DCCD has only been shown for stimulation of the ATP driven reduction of NAD^+ by succinate. For any given concentration of DCCD, the rate of reduction of NAD^+ is increased by oligomycin up to a total concentration of 0.5nmole of (DCCD+oligomycin)/mg protein. These results are interpreted by the authors as showing that the sites of DCCD and oligomycin inhibition are close enough to exclude the approach of a second inhibitory molecule, or that the two inhibitors share a common site. Aurovertin, in conjunction with oligomycin gives a rate less than that provided by oligomycin alone, suggesting that aurovertin acts at a separate site to oligomycin and to DCCD.

CHAPTER 2 INHIBITION OF ENERGY LINKED REACTIONS IN RAT
LIVER MITOCHONDRIA BY DCCD

2.1 Introduction

DCCD inhibits a range of energy linked reactions in mitochondria. In general, DCCD behaves in a similar manner, and at comparable levels, to the fungal antibiotic oligomycin (10). Both DCCD and oligomycin inhibit the uncoupler stimulated ATPase, ATP/Pi exchange, ATP dependant transhydrogenase, energy linked reduction of NAD by succinate, and ADP stimulated respiration in ETP_H and beef heart mitochondria (138, 139). The effects of oligomycin and DCCD are additive (139). Unlike oligomycin, the effects of DCCD cannot be reversed by repeated washing with phospholipid (139). The reactive nature of DCCD (140) leads one to believe that the inhibitor forms a covalent bond with some molecule in the mitochondrial membrane which is concerned with energy conservation reactions. The value of such an inhibitor lies in the possibility of covalently labelling a specific site which can be shown to be involved in the synthesis of ATP. Cattell *et al.* (78) have shown that beef heart submitochondrial particles, treated with ^{14}C DCCD at levels giving sub-maximal inhibition of ATP dependant reactions, retain 90% of the applied radiolabel. They have further shown that this bound radioactivity is associated with a chloroform/methanol soluble proteolipid with a molecular weight of 10,000 daltons.

Chloramphenicol sensitive incorporation in vitro of amino acids into the chloroform-methanol soluble proteins of rat liver mitochondria has been reported by Beattie (95). In vivo incorporation of amino acids into the proteolipid fraction of rat liver mitochondria has been demonstrated by Kuzela (96) and Kadenbach (97). Amino acid incorporation into proteolipids has also been shown to occur in the biogenesis of yeast mitochondria (57, 76, 93). The similarities between the products of mitochondrial protein synthesis and the DCCD binding protein in beef heart mitochondria may be due to identity of one of the mitochondrial products with the "Beechey protein", although DCCD binding and chloramphenicol sensitive incorporation have not been shown in the same system.

2.2. Methods and Materials

Sterile preparation of rat liver mitochondria

Rats were killed by stunning and partial exsanguination. The abdomen was washed with 90% ethanol, and the liver dissected out and washed in three changes of sterile 250mM sucrose, and transferred to a sterile, tared, beaker. Approximately 15g of liver were homogenised in 40 ml of sterile 250mM sucrose 1mM EDTA, with 2 to 3 passes of a glass-teflon homogeniser. The homogenate was centrifuged twice at 800g for 20 minutes, the pellets being discarded. The supernatant was centrifuged at 9,000g for 10 minutes, and the supernatant and loosely packed pellet of microsomes discarded. The brown hard packed pellet of mitochondria was resuspended in 250mM sucrose 1mM EDTA in a sterile

homogeniser, and centrifuged at 9,000g, the supernatant and pink layer of microsomes again being discarded. The mitochondrial pellet was washed again, and finally resuspended in a small volume of 250mM sucrose 1mM EDTA. All steps were carried out at 0°C. All glassware and dissecting instruments were sterilised by heating at 160°C overnight, or by flaming in ethanol.

Protein estimation

Mitochondrial protein was estimated by the biuret method of Gornall *et al.* (141), proteolipid was estimated by the Folin method as modified by Hess and Lewin (142).

Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis in 10% gels was essentially carried out by the method of Weber and Osbourne (143). Samples were dissolved at 1mg/ml in 0.1M phosphate buffer, 3% SDS, 5mM DTT, 5% mercaptoethanol, pH 10.

Gels were stained in 0.25% Coomassie brilliant blue in 45% methanol, 10% acetic acid, and destained in the same solvent. Gels containing radioactivity were sliced into 1mm sections and dissolved in 1ml of hydrogen peroxide at 50°C overnight.

Counting

Counting of ^3H and ^{14}C was carried out in a Packard Tricarb scintillation counter using a Triton X-100/toluene/butyl PBD scintillation cocktail (144).

Assays

Polarography was carried out according to the method

of Nijs (145). ATPase activity was measured by the method of Pullman (146) in a volume of 1ml at 30°C. ATP driven reduction of NAD by succinate and ATP driven energy linked nicotinamide nucleotide transhydrogenase were assayed by the methods of Low and Vallin (147) and Danielson and Ernster (148) as modified by Beechey (149).

Incubation of mitochondria with DCCD

Membranes were incubated with DCCD in 250mM sucrose, 10mM Tris-sulphate pH 7.4, 1mM ATP, 1mM succinate. ¹⁴C DCCD treated membranes were washed with 250mM sucrose, 10mM Tris-sulphate pH 7.4 as described by Cattell *et al.* (78). Extraction of the DCCD treated mitochondria with chloroform/methanol and purification of the proteolipid fraction was performed as described by Cattell *et al.* for beef heart mitochondria (78).

Amino Acid incorporation

In vitro incorporation of amino acids into rat liver mitochondria was carried out according to the procedure of Beattie (84). 1.25 µCi of a ³H (U) amino acid mixture (Chlorella protein hydrolysate obtained from the Radiochemical Centre, Amersham) was added per mg of mitochondrial protein. Bacterial contamination was estimated by plating out aliquots of the mitochondrial suspension onto nutrient agar plates and incubating at 30°C for 3 days. In vivo incorporation of ³H leucine (Radiochemical Centre, Amersham) was carried out as described by Kuzela *et al.* (96).

Materials

All chemicals were analytical reagent grade. Organic solvents were redistilled before use. ^{14}C -DCCD was prepared by Dr. P.A. Haarthoon (150). Cycloheximide and chloramphenicol were purchased from Sigma. ^3H -leucine and ^3H -Chlorella hydrolysate was purchased from the Radiochemical Centre, Amersham.

2.3. Results

Effect of DCCD on partial reactions

ADP stimulated respiration in rat liver mitochondria is inhibited by DCCD at 5nmoles/mg of protein (Fig. 2.1). The ATP driven reduction of NAD by succinate catalysed by rat liver submitochondrial particles is 90% inhibited by DCCD at 1.4nmoles/mg protein as illustrated in Fig. 2.2. The results presented in Fig. 2.3 show the effect of DCCD on the energy linked nicotinamide nucleotide transhydrogenase driven by ATP in rat liver submitochondrial particles. As found by Robertson (139) in beef heart mitochondria, higher concentrations of DCCD are required to inhibit this reaction than either the ATPase or ATP driven reduction of NAD by succinate. Transhydrogenase is maximally inhibited by 3nmoles/mg protein, while the Mg^{2+} ATPase in submitochondrial particles is inhibited by 1nmole/mg protein, as shown in Fig. 2.5. The ATPase reaction in rat liver mitochondria requires 10nmoles DCCD/mg protein for maximal inhibition.

Binding of ^{14}C DCCD

The binding of ^{14}C DCCD to rat liver submitochondrial

Fig 2.1

INHIBITION OF ADP STIMULATED RESPIRATION BY DCCD

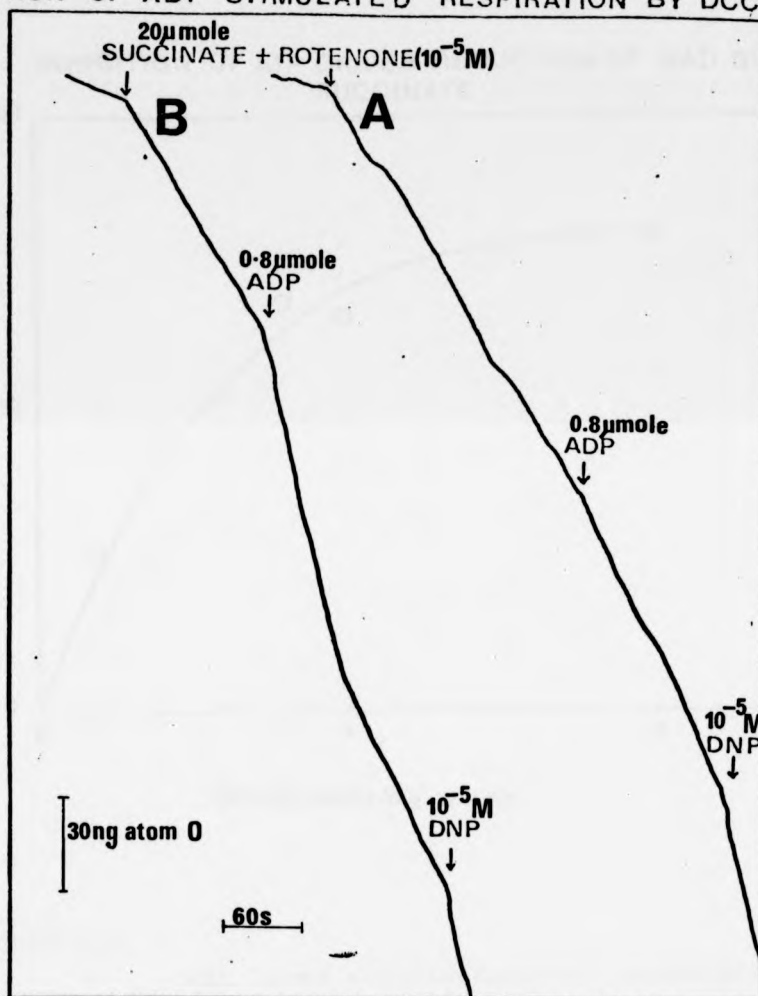


FIGURE 2.1.

Rat liver mitochondria (4mg) were incubated with (A) or without (B) 20 nmoles DCCD in a Clarke oxygen electrode for 15 minutes at 30°C. (Succinate+ rotenone), ADP and DNP were added where indicated.

Fig 2.2

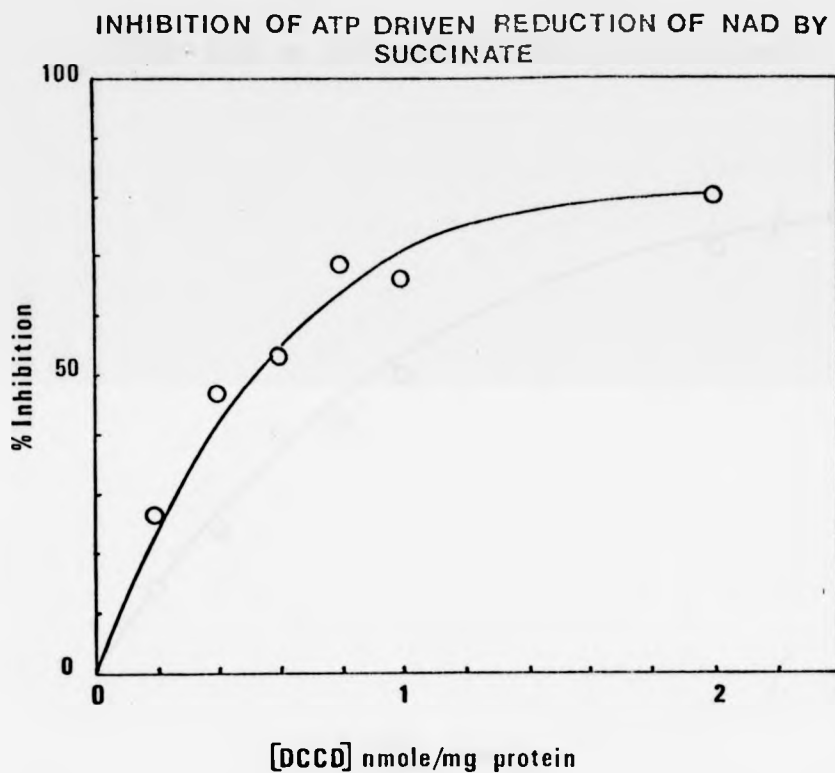


FIGURE 2.2.

Rat liver submitochondrial particles were incubated with varying amounts of DCCD at 0°C for 18 hours. 1mg SMP was assayed according to the method of Low and Vallin (147) as modified by Beechey (149).

Fig 2.3

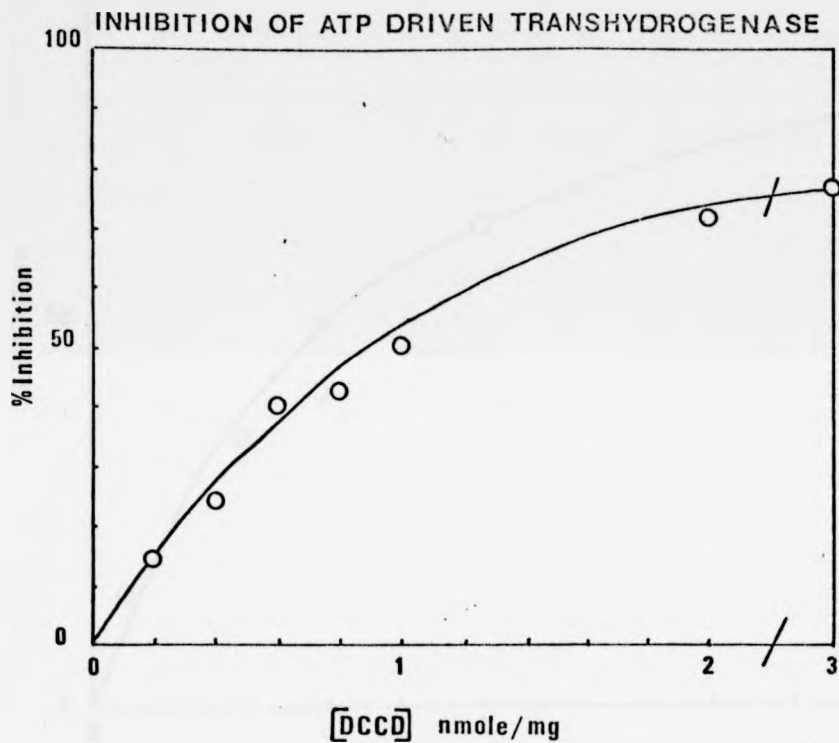


FIGURE 2.3.

Rat liver submitochondrial particles were incubated with varying amounts of DCCD at 0°C for 18 hours. 1mg SMP was assayed by the method of Danielson and Ernster (148) as modified by Beechey (149).

Fig 2.4

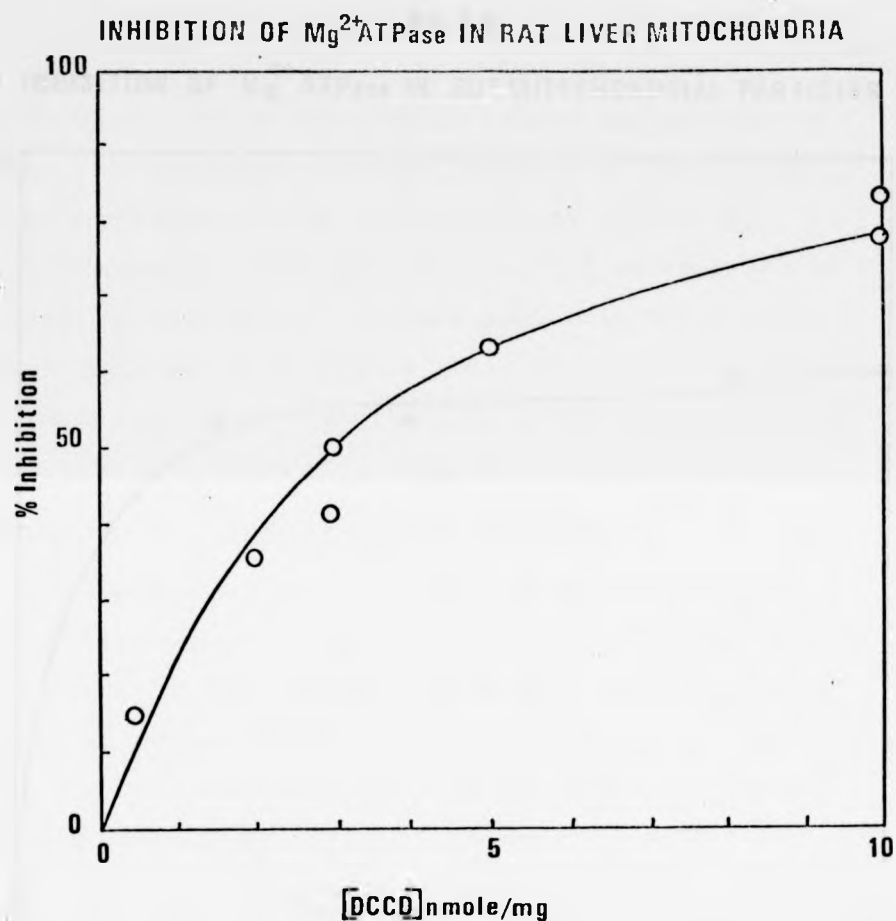


FIGURE 2.4.

Rat liver mitochondria were incubated with varying amounts of DCCD at 0°C for 18 hours. 100 μg mitochondria were assayed for ATPase activity by the method of Pullman (146).

Fig 2.5

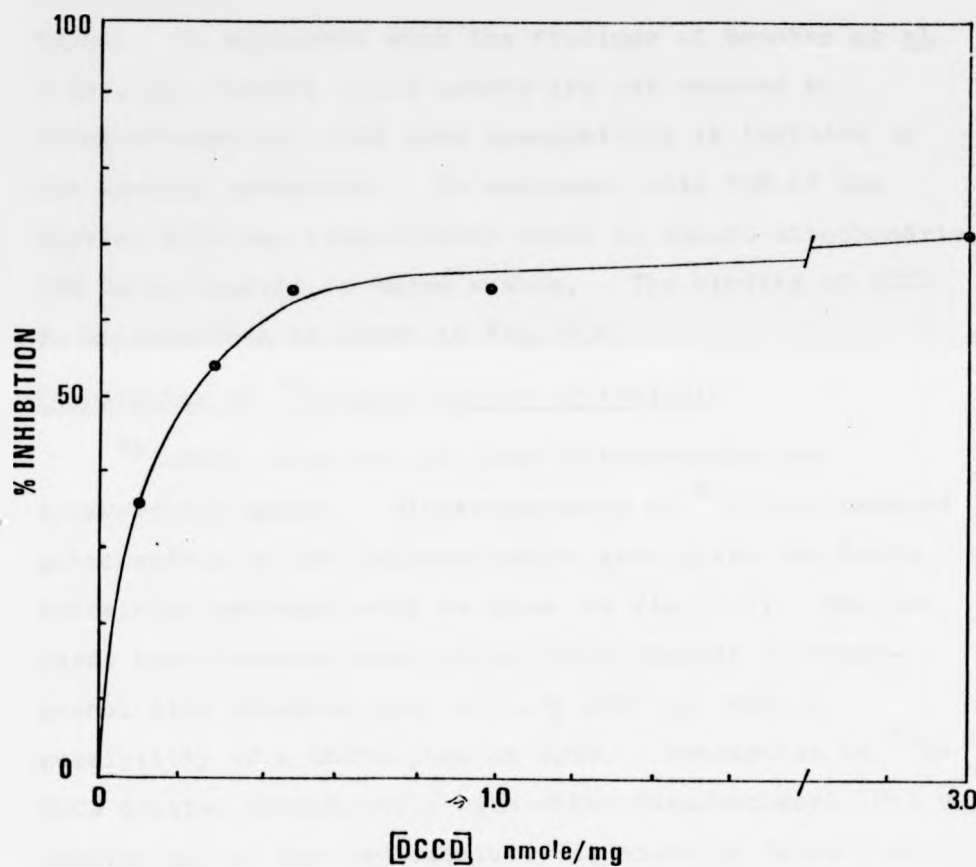
INHIBITION OF Mg^{2+} ATPase IN SUBMITOCHONDRIAL PARTICLES

FIGURE 2.5.

Rat liver submitochondrial particles were incubated with varying amounts of DCCD at 0°C for 18 hours. $100\mu\text{g}$ submitochondrial particles were assayed for ATPase activity by the method of Pullman (146).

accompanied by a corresponding difference in ^{14}C DCCD binding, as shown in Table 2.1. Maximum binding of ^{14}C DCCD occurs at less than 1nmole/mg protein, at which concentration approximately 70% of the applied counts are covalently bound. In agreement with the findings of Beechey *et al.* (138), the tightly bound counts are not removed by repeated washing, even when phospholipid is included in the washing procedure. In contrast, only 10% of the applied DCCD was irreversibly bound to intact mitochondria, 90% being removed in three washes. The binding of DCCD to mitochondria is shown in Fig. 2.6.

Preparation of ^{14}C -DCCD binding proteolipid

^{14}C -DCCD bound to rat liver mitochondria was irreversibly bound. Electrophoresis of ^{14}C -DCCD treated mitochondria in SDS polyacrylamide gels gives two bands containing radioactivity as shown in Fig. 2.7. The two bands have relative mobilities (with respect to bromophenol blue tracking dye) of 1.16 and 1.0, with a possibility of a third peak at 0.69. Extraction of ^{14}C -DCCD treated mitochondria with chloroform/methanol (2:1 v/v) removes all of the radioactivity as shown in Table 2.2., but only half the protein. Most of the protein may be removed from the extract by washing with 0.2 volumes of water, with negligible diminution of the ^{14}C content. Precipitation of the extracted proteolipid by cold ether results in loss of about half of the counts into the supernatant in contrast to the findings of Cattell *et al.* (78) in beef heart mitochondria. Polyacrylamide gel electrophoresis of the ether precipitated proteolipid in

TABLE 2.1. Binding of ^{14}C DCCD to rat liver submitochondrial particles

Concentration of DCCD (nmoles/mg)	^{14}C (dpm)	dpm Bound	% Bound
1.70	313,680	213,218	65.8
0.27	34,573	23,318	68.0

SMP were incubated with ^{14}C DCCD at 0 - 4°C for 18 hours in 250mM Sucrose/10mM Tris-sulphate/5mM Magnesium sulphate/1mM ATP/1mM Succinate, pH 7.4. Treated particles were washed in 250mM Sucrose/10mM Tris-sulphate, pH 7.4. Samples were counted in Toluene/Triton X-100/Butyl PBD.

Fig 2.6

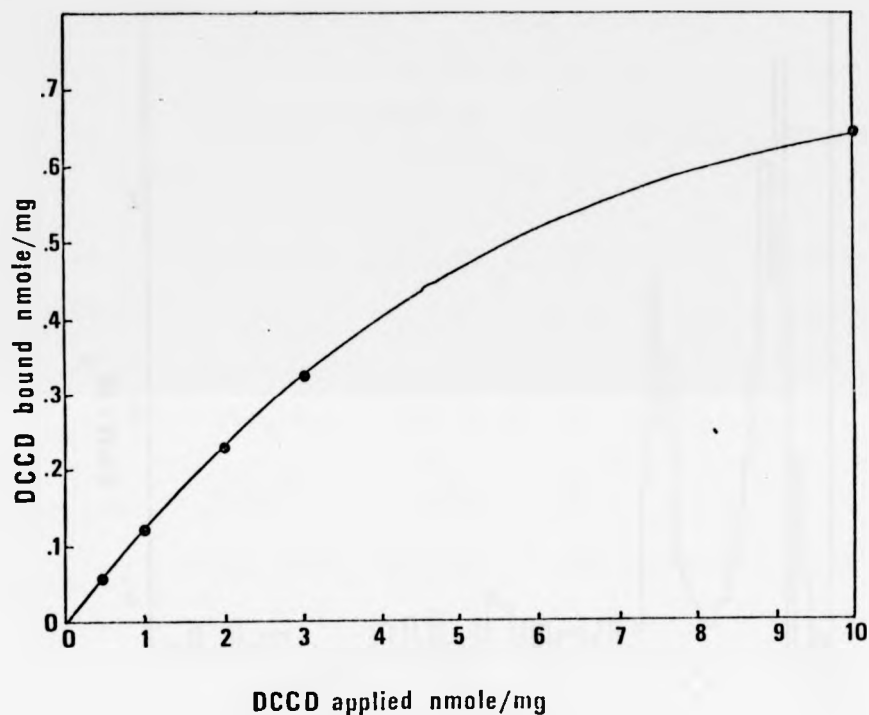
BINDING OF ^{14}C DCCD TO RAT LIVER MITOCHONDRIA

FIGURE 2.6.

^{14}C DCCD was incubated with 2mg mitochondrial protein at 0°C for 18 hours in 5ml 250mM Sucrose, 10mM Tris-acetate pH 7.5. The mitochondria were centrifuged at 14,000g and the radioactivity in the pellet determined by liquid scintillation in triton/toluene/butyl PBD.

Fig 2.7

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS
RAT LIVER MITOCHONDRIA

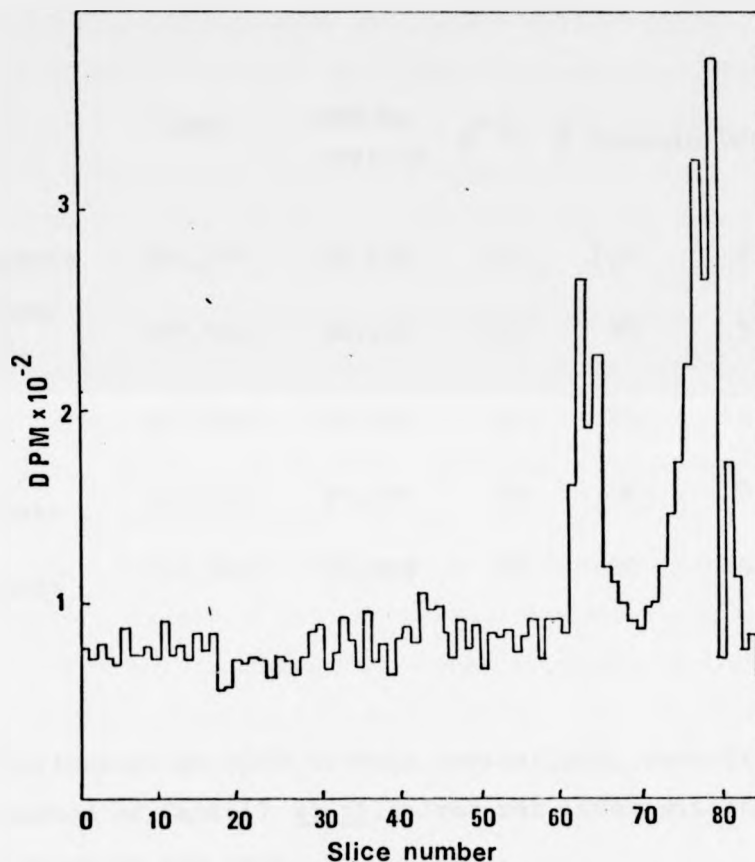


FIGURE 2.7.

¹⁴C DCCD was incubated with rat liver submitochondrial particles at 1nmole/mg for 18 hours at 0°C. The particles were washed three times and dissolved at 1mg/ml in 3% SDS, 5% mercaptoethanol, 100mM phosphate pH 10 by boiling for 2 minutes. 150μg of solubilised SMP were electrophoresed on 7.5% polyacrylamide gels as described by Weber and Osbourne (143). Gels were sliced into 1mm sections and dissolved in 1ml 6% hydrogen peroxide at 60°C. Radioactivity was determined by liquid scintillation in triton/toluene/butyl PBD.

TABLE 2.2. Purification of proteolipid

Sample	DPM	DPM/mg protein	% ¹⁴ C	% Protein	DCCD/mg
Mitochondria	376,714	16,684	100	100	2.5
Chloroform/ Methanol extract	407,160	36,484	108	49	5.6
Washed Extract	341,752	59,956	91	25	9.2
Ether Precipitate	103,293	59,024	28	8	9.0
Ether supernatant	152,500	33,888	40	20	5.2

Purification of DCCD binding proteolipid, according to the method of Cattell et al., from rat liver mitochondria. Details given in the text.

SDS shows that the ^{14}C migrates in two bands with mobilities of 0.95 and 0.68 as shown in Fig. 2.8. Electrophoresis of standard proteins (cytochrome c, pyruvate kinase, ribonuclease and lactic dehydrogenase) shows that these mobilities correspond to molecular weights of 31,000 and 11,000 respectively. The front running band with a mobility of 1.16 has disappeared and may correspond to the radioactivity remaining in the ether supernatant. Thin layer chromatography of the chloroform methanol extracts from ^{14}C -DCCD treated mitochondria shows that the ^{14}C labelled material is roughly divided into two equal parts, a ninhydrin staining portion which remains at the origin, and one which does not stain with ninhydrin and migrates in Benzene/Ethyl Acetate (9:1) on Alumina plates. Thin layer chromatography of the precipitate and supernatant at the ether precipitation step shows that only this migrating band remains in the supernatant and that the ninhydrin staining, non-migrating portion only is found in the precipitate. Thin layer chromatograms of the ether supernatant with DCCD and DCU standards are shown in Fig. 2.9. The radiolabel is not associated with phospholipid as shown by chromatography with DCCD labelled standards.

Amino Acid Incorporation

Amino acid incorporation in vitro by the method of Beattie (84), was shown to be substantially free from bacterial contamination, less than 500 bacteria per ml of incubation mixture being routinely found by viable count. The incorporation had only a very slight lag phase, as illustrated in the time course of incorporation in Fig. 2.10, indicating negligible contribution by

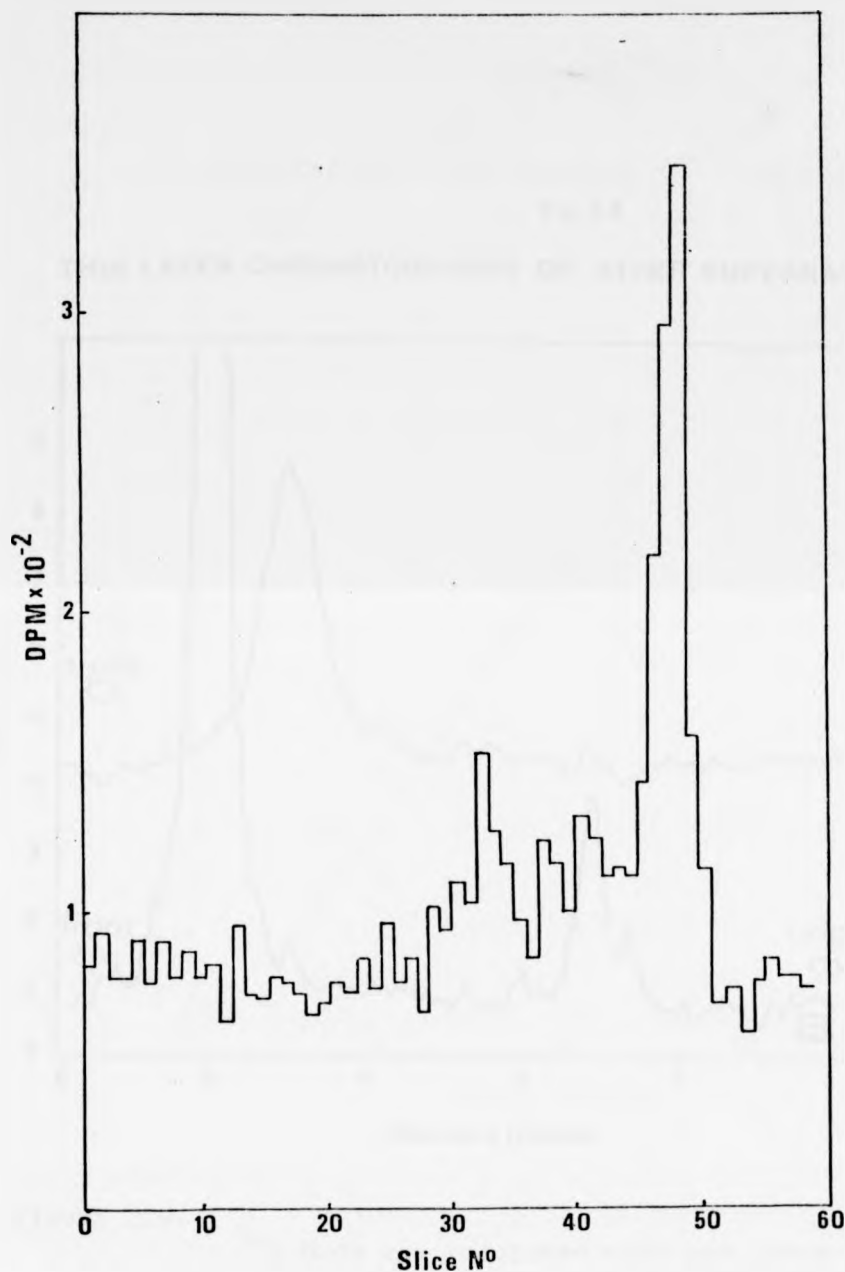
ELECTROPHORESIS OF ^{14}C DCCD-PROTEOLIPID

FIGURE 2.8.

^{14}C DCCD was incubated with rat liver sub-mitochondrial particles at 1nmole/mg for 18 hours at 0°C. The particles were washed three times and proteolipid prepared by the method of Cattell *et al.* (78). Proteolipid was loaded onto the gels at 150µg/gel and run according to Weber and Osbourne (143). Gels were sliced and counted as described in Methods.

Fig 2.9

THIN LAYER CHROMATOGRAPHY OF ETHER SUPERNATANT

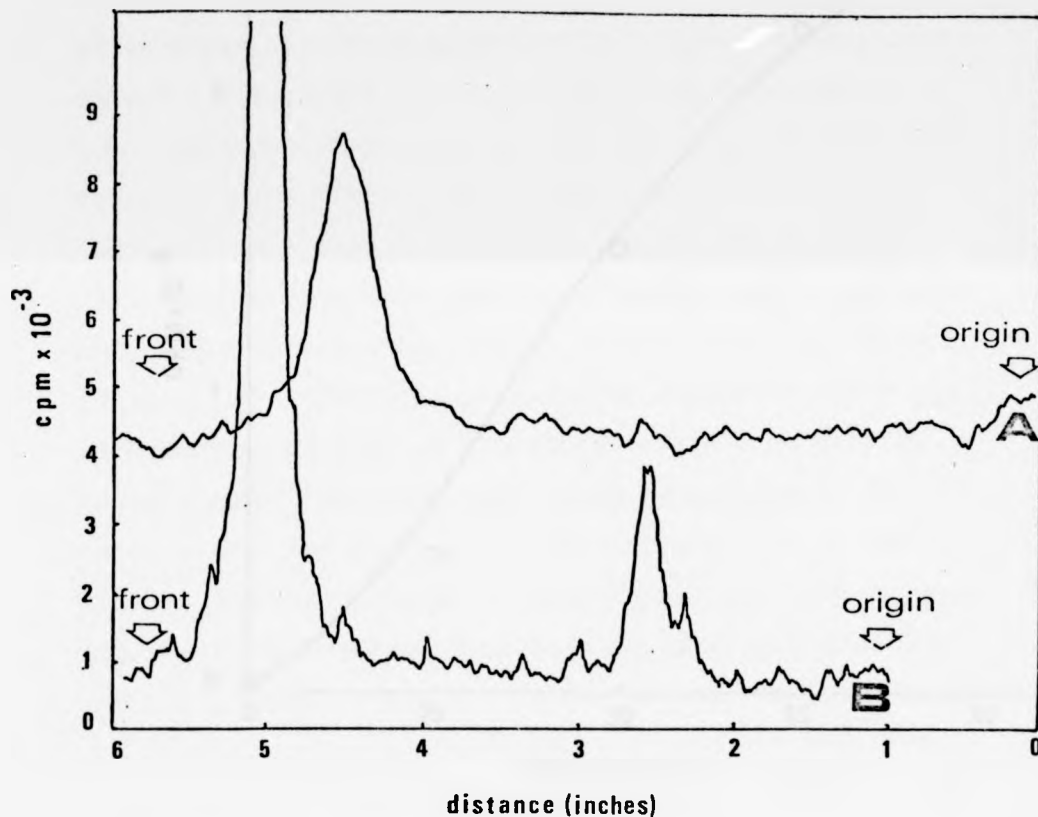


FIGURE 2.9.

^{14}C DCCD was incubated with rat liver sub-mitochondrial particles at 1nmole/mg for 18 hours at 0°C . Proteolipid was prepared as described by Cattell *et al.* (78). Radioactivity remaining in the supernatant after ether precipitation of proteolipid was run on aluminium oxide TLC plates in Benzene/Ethyl acetate (9:1 v/v).

A Supernatant after ether precipitation of proteolipid

B Standards, DCCD (R_f 0.9) and DCU (R_f 0.36).

Fig 2.10

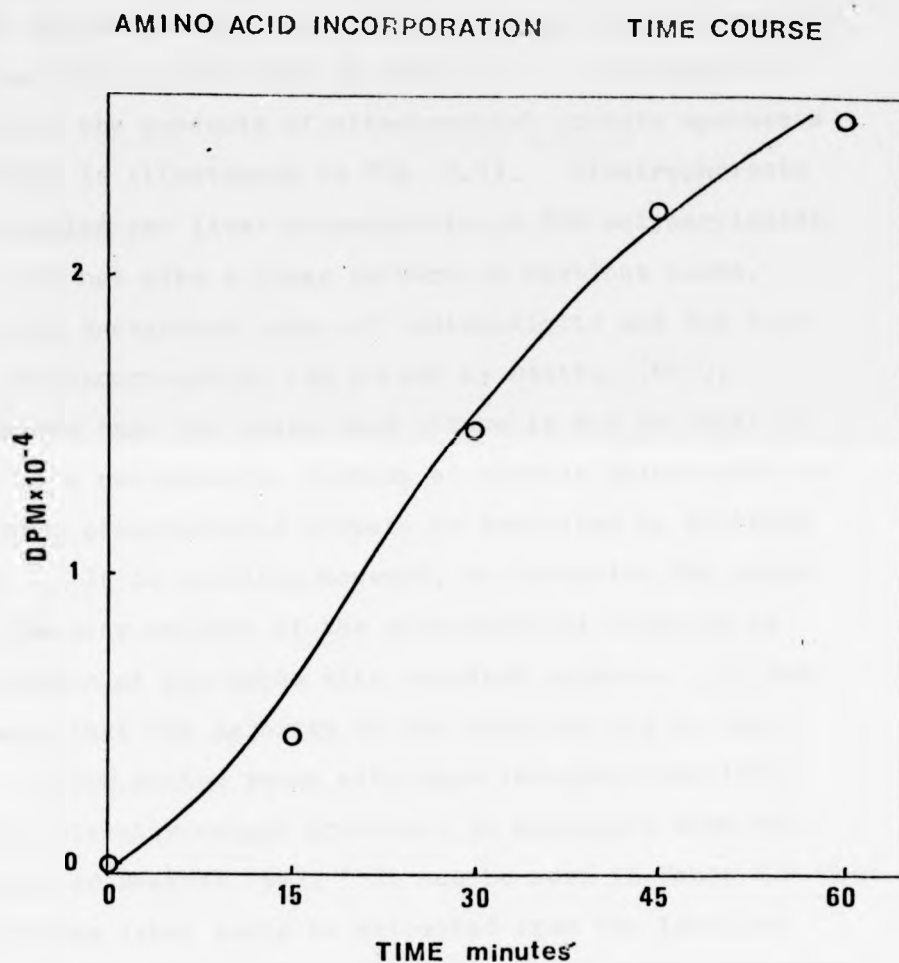


FIGURE 2.10.

Incorporation of ^3H -amino acids into rat liver mitochondria in vitro was performed by the method of Beattie (84). Aliquots of the incorporation medium were removed at the times shown and incorporation terminated by addition of an equal volume of 10% TCA. The TCA precipitate was washed with cold amino acids (5mM) and radioactivity determined by liquid scintillation in triton/toluene/butyl PBD.

bacteria to the uptake of amino acids. The incorporation of the amino acid mixture into mitochondria was found to be chloramphenicol sensitive and cycloheximide resistant as shown by the data in Table 2.3. An attempt to identify the products of mitochondrial protein synthesis in vitro is illustrated in Fig. 2.11. Electrophoresis of labelled rat liver mitochondria in SDS-polyacrylamide gels did not give a clear pattern of distinct bands. The high background level of radioactivity and the high rate of incorporation (as found by Beattie (84)), indicates that the amino acid uptake is due at least in part to a non-specific binding of certain amino acids to existing mitochondrial protein as described by Hochberg (151). It is possible however, to determine the range of molecular weights of the mitochondrial products by comparison of the bands with standard markers. It can be seen that the majority of the products lie in the 20 - 45,000 dalton range with some incorporation into lower molecular weight proteins, in agreement with the results of Beattie (94). It can be seen in Table 2.4 that 20% of the label could be extracted from the labelled mitochondria by chloroform/methanol (2:1) under the same conditions as preparation of the DCCD-binding proteolipid. In an attempt to overcome the problems of both bacterial contamination and non-specific binding, the in vivo incorporation of ^3H leucine into cycloheximide treated rats was studied. Rats were injected intraperitoneally with cycloheximide to inhibit cytoplasmic protein synthesis, followed by injection of $7\text{mCi } ^3\text{H}$

TABLE 2.3. Inhibitor sensitive incorporation of amino acids

System	Dpm/mg Protein
Normal	25,133
plus Cycloheximide	19,609
plus chloramphenicol	2,694
Stopped at zero time	299

Sterile (300 - 400 bacteria/ml) mitochondria were incubated at 30°C in a shaking water bath. Incorporation medium according to Beattie (34) contained 50mM Tris-chloride/10mM Sodium phosphate/154mM potassium chloride/5mM Magnesium chloride/10mM Succinate/2mM ADP/2mM EDTA, pH 7.2. Mitochondria were added to 1mg/ml. Chloramphenicol concentration was 1 μ M, cycloheximide 10 μ M.

POLYACRYLAMIDE GEL ELECTROPHORESIS; RAT LIVER
MITOCHONDRIA

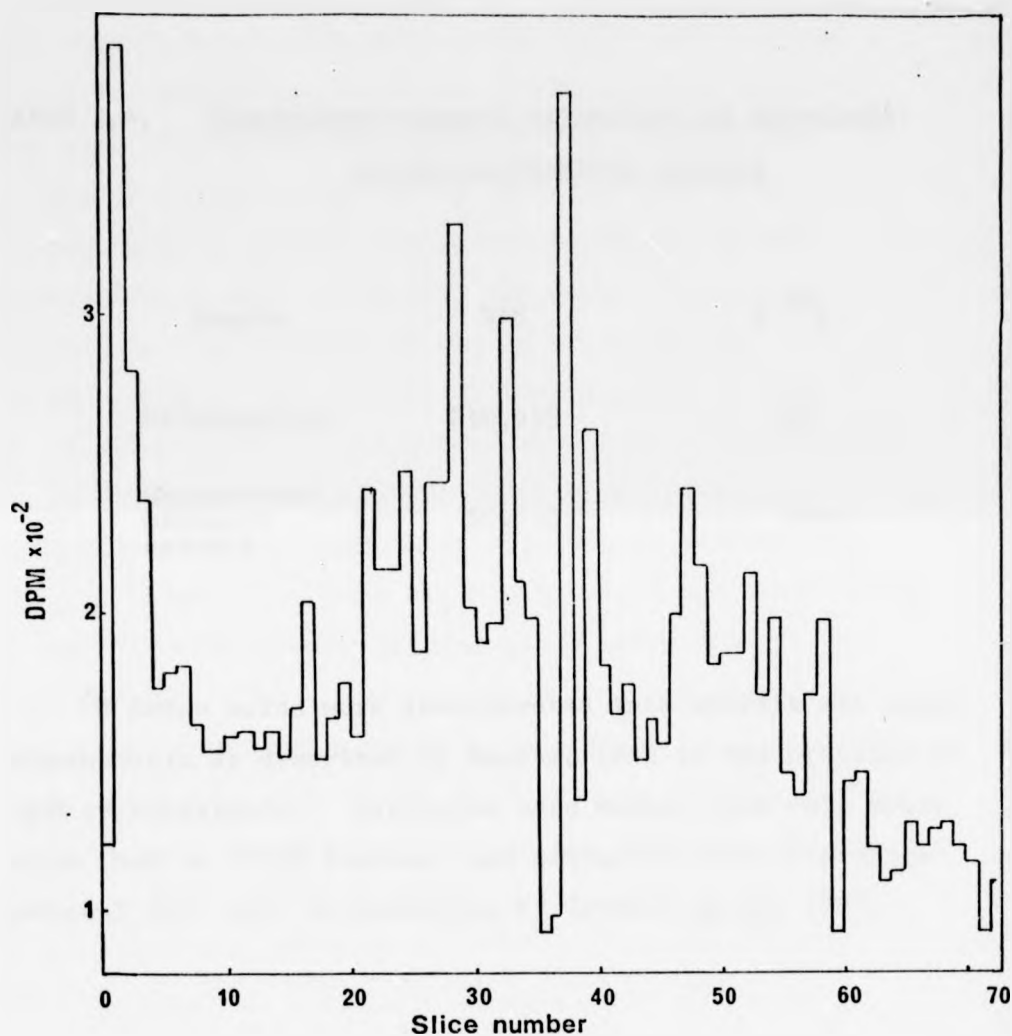


FIGURE 2.11.

Incorporation of ^3H amino acids into rat liver mitochondria in the presence of cycloheximide was performed as described by Beattie (84). Incorporation was terminated by addition of an equal volume of 10% TCA. The TCA precipitate was washed with cold amino acids (5mM, each amino acid) and dissolved in 3% SDS, 5% mercaptoethanol, 100mM phosphate buffer pH 10. Polyacrylamide gel electrophoresis was performed by the method of Weber and Osbourne (143). Gel slices (1mm) were dissolved in 1ml 6% hydrogen peroxide and counted in 9ml triton/toluene/butyl PBD scintillant.

TABLE 2.4. Chloroform-methanol extraction of mitochondrially synthesised protein

Sample	DPM	% ^{14}C
Mitochondria	230,435	100
Chloroform/ methanol extract	50,132	21.8

^3H Amino acids were incorporated into sterile rat liver mitochondria as described by Beattie (84) in the presence of $10\mu\text{M}$ cycloheximide. Particles were washed with cold amino acids (5mM in 250mM sucrose) and extracted with chloroform-methanol (2:1 v/v) as described by Cattell et al. (78).

leucine/Kg body weight. A 'cold' leucine chase was employed to dilute non-incorporated label and complete partially synthesised peptides. Mitochondria prepared from rats treated in this manner showed a high level of incorporation of tritium as shown in Table 2.5. Electrophoresis of the mitochondrial protein in SDS-polyacrylamide gels shows that the major product, in contrast to the products of the in vitro system, is a low molecular weight protein with a relative mobility of 1.0 (Fig. 2.12). The molecular weight of the protein may be calculated to be approximately 10,000 daltons. Extraction of the mitochondria with chloroform/methanol (2:1 v/v) under the same conditions as in the preparation of the ^{14}C DCCD binding proteolipid removes 20% of the counts (Table 2.5). Electrophoresis of the extracted membranes in SDS-polyacrylamide gels shows that the low molecular weight protein has disappeared, and only the proteins with molecular weights in the range 32,000 - 80,000 daltons remain as shown in Fig. 2.13. In order to determine the identity of the low molecular weight peptides found in these preparations, rat liver mitochondria were labelled in vitro with ^3H -leucine in the presence of cycloheximide. Incubation of submitochondrial particles, derived from ^3H labelled mitochondria, with ^{14}C DCCD resulted in binding of 70% of the DCCD. Polyacrylamide gel electrophoresis of the double labelled submitochondrial particles shows that a peptide with a molecular weight of 10,000 daltons is labelled with both ^3H and ^{14}C as illustrated in Fig. 2.14.

TABLE 2.5. Chloroform-methanol extraction of mitochondrially synthesised protein

	Protein (mg)	^3H cpm	^3H cpm/mg
SMP	40	364,000	9,100
Chloroform- methanol extract	16	73,000	4,600

A 300g Wistar rat was injected with 20mg cycloheximide. 15 minutes later 2mCi ^3H leucine were injected inter-peritoneally. After two hours the rat was injected with cold leucine and killed. Rat liver mitochondria were prepared as described in Methods. Chloroform-methanol extraction was performed as described by Cattell et al. (78).

Fig 2.12

ELECTROPHORESIS (RAT LIVER MITOCHONDRIA)

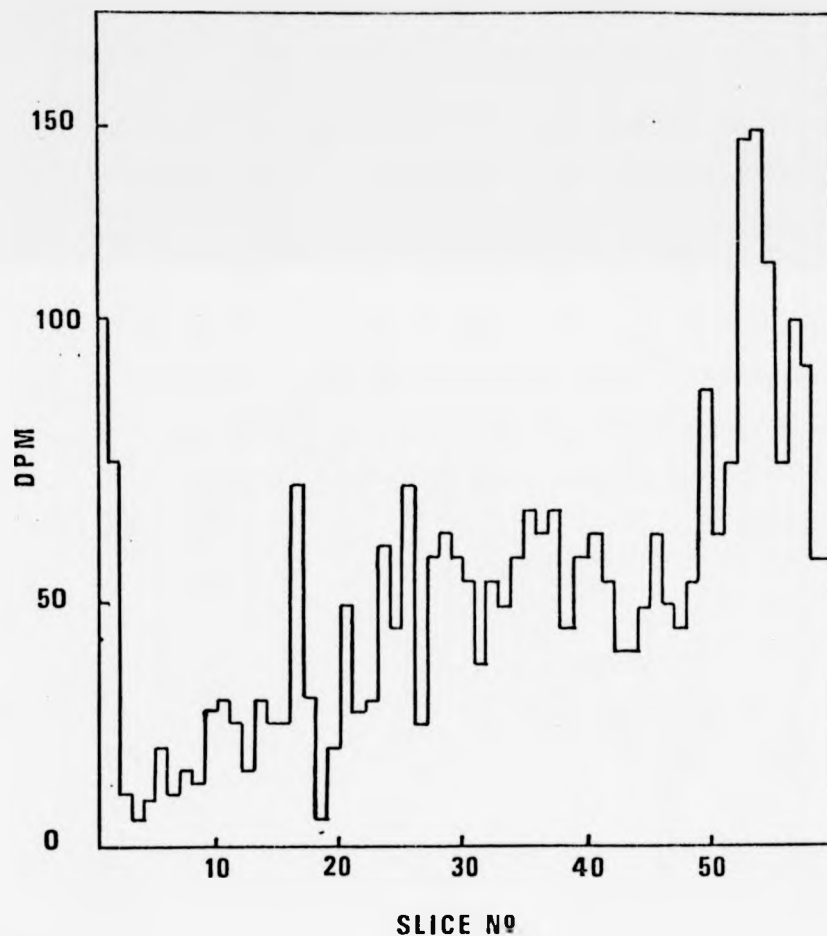


FIGURE 2.12.

Incorporation of ^3H leucine into rat liver mitochondria in the presence of cycloheximide was performed by the method of Kuzela (96). Submitochondrial particles were dissolved in 3% SDS, 5% mercaptoethanol, 100mM phosphate buffer pH 10. Gel electrophoresis was performed according to Weber and Osbourne (143) 1mM gel slices were dissolved in 1ml 6% hydrogen peroxide and counted in 9 ml triton/toluene/butyl PBD scintillation cocktail.

FIGURE 2.13.

Incorporation of ^3H leucine into rat liver mitochondria was performed according to Kuzela (96). Chloroform-methanol extraction of submitochondrial particles was carried out as described by Cattell (78). Chloroform-methanol extracted particles were dissolved in 3% SDS, 5% mercaptoethanol, 100mM phosphate pH 10. Electrophoresis was carried out as described by Weber and Osbourne (143), gel slices were dissolved in 6% hydrogen peroxide.

Fig 2.13

CHLOROFORM METHANOL EXTRACTED PARTICLES

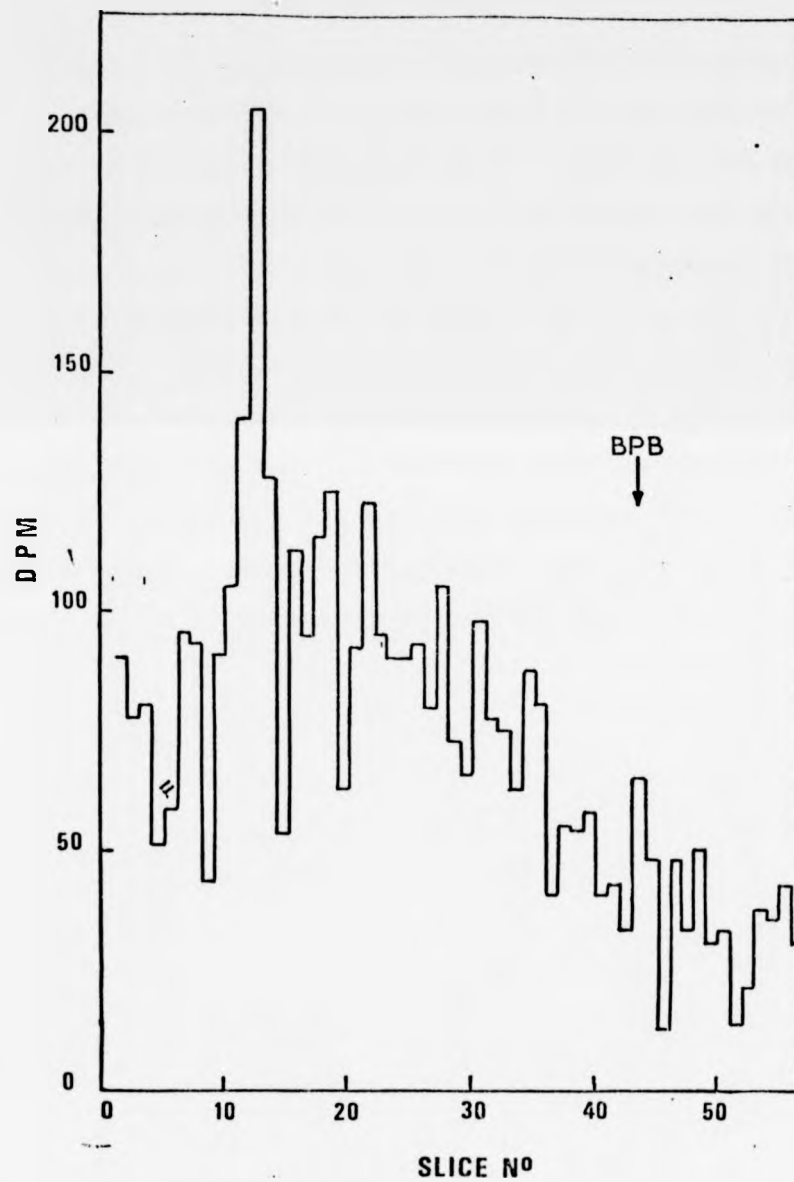
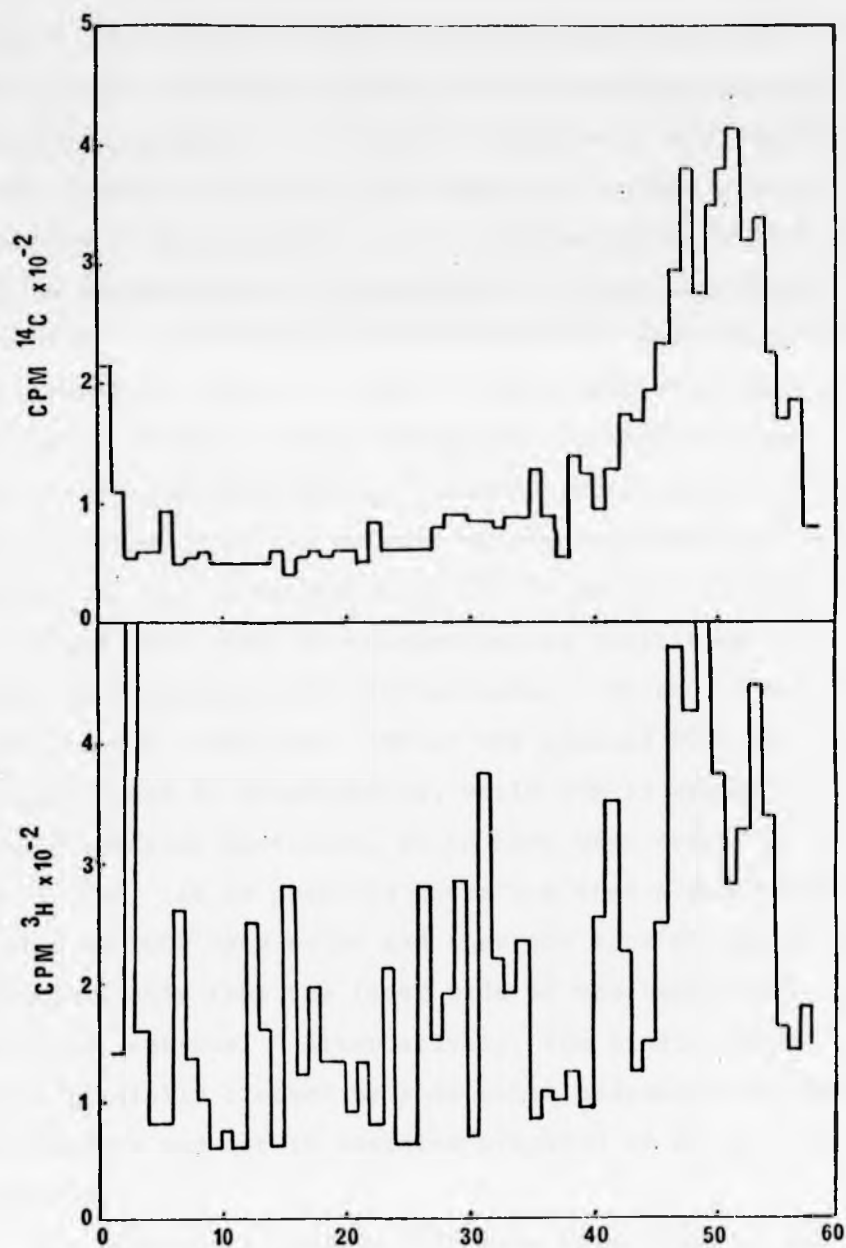


Fig. 2.14.

Double labelling of the DCCD binding protein

Incorporation of ^3H leucine in the presence of cycloheximide into rat liver mitochondria was performed as described by Kuzela (96). Submitochondrial particles were prepared from the washed mitochondria as described in Methods. ^{14}C DCCD was incubated with ^3H -submitochondrial particles at 0°C for 18 hours. The washed SMP were dissolved in 3% SDS, 5% mercaptoethanol, 100mM phosphate pH 8.5. Electrophoresis in 7.5% polyacrylamide gels was performed as described by Weber and Osbourne (143). 1mM gel slices were dissolved in 1ml 6% hydrogen peroxide and counted by liquid scintillation in 9ml triton/toluene/butyl PBD. Spillover of ^{14}C into ^3H was corrected for by reference to known standards.

Fig 2.14



2.4 Discussion

DCCD inhibits ATP dependant reactions in rat liver mitochondrial particles at levels comparable with those found for electron transfer particles from beef heart (138). The ATPase in intact mitochondria, however, requires ten times the concentration of DCCD as submitochondrial particles. A tenfold increase in the sensitivity is the opposite effect to that expected, as the site of action would be expected to be in the membrane (139) and thus be concentrated by preparation of submitochondrial particles. One mechanism explaining this phenomenon is a permeability barrier to DCCD at the inner mitochondrial membrane. DCCD is a very lipophilic molecule and as such should pass through the inner membrane easily. It has been shown that the potency of carbodiimides is related to their hydrophobicity (101) and that DCCD is one of the most effective carbodiimides inhibiting oxidative phosphorylation in bacteria. It should be noted, however, that only 10% of the applied DCCD is strongly bound to mitochondria, while 70% is bound in submitochondrial particles, which have been evoked by sonication. It is possible therefore that a permeability barrier to DCCD does exist and that the site of action is accessible only from the inner side of the inner mitochondrial membrane. Alternatively, the binding site may be partially blocked by some other component in intact mitochondria and not in vesicles prepared by sonic disruption.

Two components binding DCCD have been found in rat

liver mitochondria. This does not conflict with the results obtained by Cattell et al. (78) and Broughall (152) in beef heart mitochondria. Cattell et al. recovered 43% of ^{14}C -DCCD in the purification of DCCD-binding proteolipid but give no details of the remaining 57% ^{14}C . Broughall recovered 34% of the ^{14}C DCCD as proteolipid, but found that 27% of the counts remained in the supernatant after ether precipitation. Both the binding data and the results of proteolipid preparation indicate two binding sites for DCCD in rat liver mitochondria. Analysis of the binding of ^{14}C DCCD by Scatchard plot (Fig. 2.14) gives two straight lines indicating two binding sites, a tightly binding site ($K_D = 7.8 \times 10^{-5}\text{M}$) and a loose binding site ($K_D = 1.25 \times 10^{-3}\text{M}$). Examination of SDS-polyacrylamide gels of ^{14}C DCCD treated mitochondria shows three bands containing ^{14}C , of which the front running band (relative mobility 1.16) is absent in similar gels of proteolipid precipitated by ether, as shown in Figs. 2.7 and 2.8. Thin layer chromatography of the proteolipid fraction precipitated by cold ether shows that there is only one band, staining with ninhydrin, which remains at the origin, but two bands are found in chromatograms of the chloroform/methanol extract. The second band, is also found in chromatograms of the supernatant remaining after ether precipitation of the proteolipid, and has the same relative mobility (0.85) as free DCCD. The second ~~component~~ binding DCCD is probably mitochondrial lipid; in aqueous preparations the DCCD is bound in lipid vesicles, but in organic solvents,

Fig 2.15

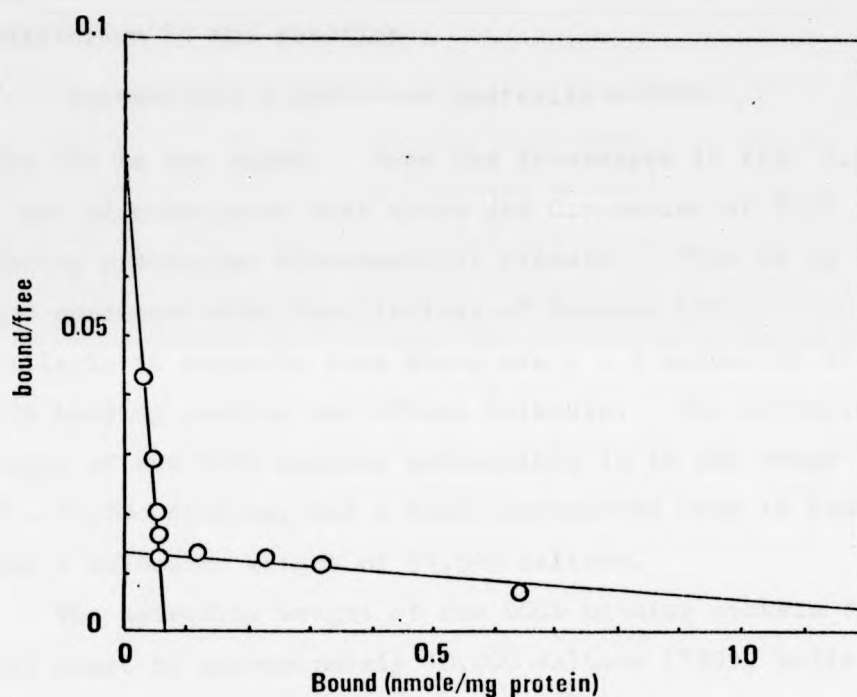
BINDING OF ^{14}C DCCD TO MITOCHONDRIA: SCATCHARD PLOT

FIGURE 2.15.

^{14}C DCCD was incubated with 2mg mitochondrial protein at 0°C for 18 hours in 5ml 250mM Sucrose, 10mM Tris-acetate pH 7.5. The mitochondria were centrifuged at 14,000g and the radioactivity in the pellet determined by liquid scintillation in triton/toluene/butyl PBD.

the DCCD is freed from the lipid.

DCCD bound to the proteolipid fraction is usually assumed to be bound covalently, but the data presented in Fig. 2.15 suggests that this is not so, although the equilibrium in the reaction



lies far to the right. From the intercepts in Fig. 2.15, it can be calculated that there are 0.7 nmoles of DCCD binding protein/mg mitochondrial protein. This is in good agreement with the findings of Beechey (71). Similarly it suggests that there are 2 - 3 molecules of DCCD binding protein per ATPase molecule. The molecular weight of the DCCD binding proteolipid is in the range 10 - 11,000 daltons, but a small associated peak is found with a molecular weight of 31,500 daltons.

The molecular weight of the DCCD binding protein from beef heart is approximately 10,000 daltons (139), while the proteolipid extracted from yeast OS-ATPase by Tzagoloff (57) has a molecular weight of 8,000 daltons. Tzagoloff has also shown that the OS-ATPase proteolipid migrates with an apparent molecular weight of 45,000 in SDS-gels, and treatment with base or organic solvents causes it to migrate with a molecular weight of 8,000. Possibly the 31,500 dalton protein in the rat liver proteolipid corresponds to this 45,000 dalton product in the yeast OS-ATPase complex, although it is not affected by organic solvents. The effect of base on the 31,500 dalton protein cannot be determined as the carbodiimide-protein bond is base labile.

The biogenesis of the chloroform extractable proteolipids has been examined both in vitro and in vivo. In the former case, chloramphenicol sensitive incorporation of amino acids was mainly into proteins with molecular weights of 20 - 45,000 daltons, in agreement with the results obtained by Beattie (94). The major peak in the rather diffuse band covering this molecular weight range lies at about 39,000 (Beattie 40,000) daltons. Burke and Beattie have shown that the lyophilisation of rat liver mitochondria converts this 40,000 dalton band into a 10 - 14,000 dalton band (95). It is probable that this corresponds to both the 31,500 dalton band found in ^{14}C -DCCD labelled proteolipid preparations and to the 45,000 dalton band found by Tzagoloff (57, 76). 20% of the radioactive label could be removed by chloroform/methanol, but SDS-polyacrylamide gel electrophoresis of the extract was not possible as the quantities used did not give a discernable ether precipitate.

Gels of mitochondria labelled in vivo in the presence of cycloheximide indicate that the major product of mitochondrial protein synthesis is a protein with a molecular weight of 10,000 daltons, in agreement with the results of Kuzela (96). Chloroform/methanol extraction of these mitochondria removes about 20% of the radiolabel, but electrophoresis of the extract was not possible as for the in vivo experiment. Electrophoresis of the extracted membranes, however, shows that the main product of mitochondrial protein synthesis is extracted from the membrane, only bands with molecular weights in excess of 46,000

daltons remaining.

Polyacrylamide gel electrophoresis of submitochondrial particles, labelled with ^3H leucine in vivo, and treated with ^{14}C DCCD, show that the chloroform methanol soluble 10,000 molecular weight proteins which are the major product in mitochondrial biogenesis, and the binding site of DCCD, comigrate in the presence of SDS. It would seem reasonable, on the basis of the similarity between the major product of protein synthesis and the DCCD binding protein, namely their solubility in chloroform methanol and co-migration in SDS-gels, that the two proteins are in fact identical.

CHAPTER 3. SOLVENT EXTRACTION OF MITOCHONDRIA

3.1. Introduction

The effect of lipids on the properties of membrane bound enzymes is well documented (153). The delipidation of membrane bound enzymes has been effected by such diverse methods as extraction with organic solvents, detergents, bile salts and treatment with lipolytic enzymes (153). Extraction of mitochondria with organic solvents has been shown to result in the loss of respiration or phosphorylation (153 - 155). Lenaz and co-workers have shown that extraction of mitochondrial membranes with alcohols has a profound effect on the functioning of oxidative phosphorylation (154, 155). The interaction of phospholipids with lipid depleted membranes has been shown to be inhibited by alcohols (156). Alcohols have also been shown to extract phospholipids from beef heart mitochondria (157). Enzymic activities damaged by such extraction include succinoxidase and ATPase, but at low concentrations of alcohol the ATPase is rendered oligomycin insensitive (155). The effect of alcohols on these enzymes is not reversed by addition of phospholipid (158), and has been shown to be related to the hydrophobicity of the alcohol used (155).

Diethyl ether has also been used for delipidation of the ATPase and appears to be similar to long chain alcohols in that oligomycin insensitivity may be induced (155, 159), but relatively little phospholipid may be removed from beef heart mitochondria by ether extraction (157), and the ATPase activity is not decreased (159).

Broughall et al. have shown that ether extraction renders the ATPase insensitive to venturicidin and DCCD.

3.2. Methods and Materials

Preparation of mitochondria and submitochondrial particles

Rat liver mitochondria were prepared by the method of Johnson and Lardy (160). Yeast mitochondria were prepared as described by Tzagoloff (41), from a commercially available strain of Saccharomyces cerevisiae. Insect flight muscle mitochondria were prepared by the method of Van den Berg (161). Beef heart mitochondria were prepared by the method of Sanadi and Fluharty (162). Submitochondrial particles were prepared by sonic disruption of mitochondria in an M.S.E. sonicator, and sedimented by centrifugation at 100,000g.

Assay of enzymic activities and extraction with organic solvents

ATPase activity was measured as described by Pullman et al. (146), or by the method of Wakabayashi and Gunge (163). Phosphate estimation was performed as described by Fiske and SubbaRow (164). Particles were preincubated with inhibitors for 5 minutes at 30°C, except for NCCD and DCCD which were incubated at 0°C for 18 hours. Extraction of particles with organic solvents for ATPase assays were performed as described by Broughall et al. (159), the solvent being removed by aspiration and nitrogen blown through the suspension. In titrations of the organic solvents, the solvent was added directly to the mitochondrial suspension and was not removed. All extract-

ions were performed at 0°C. Protein was estimated by the biuret method of Gornall *et al.* (141).

Electron Spin Resonance

E.S.R. spectra were obtained using a Decca Radar X1 with a Newport Instruments 7 inch magnet. Samples were run in sealed glass capillary pipettes. Samples were incubated with the spin probe NCCD at 0°C for 18 hours, and with stearic acid derivatives for 5 minutes at 0°C. The NCCD treated mitochondrial preparations were washed four times in 50 volumes of sucrose-tris pH 7.5. Potassium ferricyanide (1mM) was added to all buffers to prevent reduction of the nitroxide spin probe.

Materials

Ossamycin and peliomycin were obtained from NCI via Dr. H. Schmidt. Venturicidin was a gift from Dr. I. Fleming (Glaxo Laboratories Ltd.). Trialkyl tin chlorides and DCCD were obtained from British Drug Houses Ltd. Oligomycin was purchased from Sigma Chemical Co. Ltd. and was a mixture of oligomycins A(15%) and B (85%). ^{14}C DCCD was a gift from Prof. R.B. Beechey (Shell Research Ltd.) and ^{113}Sn triethyl tin was purchased from the Radiochemical Centre (Amersham). NCCD was synthesised by P. Iannou. All organic solvents were redistilled before use, all other reagents were analytical grade.

Counting

^{14}C and ^{113}Sn were counted in a Packard Tricarb Scintillation counter in a Triton X-100/Toluene/butyl PBD scintillation cocktail (144). ^{113}Sn was counted as its

Indium decay product (165).

3.3. Results

Extraction of particles with organic solvents

It can be seen from the data presented in Table 3.1 that oligomycin sensitivity is destroyed by extraction of beef heart submitochondrial particles with a variety of organic solvents. At high concentrations of methyl acetate the ATPase activity is destroyed, and some inhibition of the ATPase is found with carbon tetrachloride but diethyl ether has no inhibitory effect on the ATPase activity. Extraction of submitochondrial particles from a variety of sources leads to loss of ATPase inhibition by rutamycin, oligomycin, venturicidin, peliomycin, ossamycin, triethyl tin, DCCD and NCCD but not aurovertin B. This phenomenon has been demonstrated in submitochondrial particles derived from Saccharomyces cerevisiae (Table 3.2), beef heart (Table 3.3) and Sarconhaga barbata flight muscle (Table 3.4).

Effect of ether extraction on inhibitor binding

Table 3.6 illustrates the inextractability of the binding site for DCCD. Particles extracted with diethyl ether will still bind ^{14}C -DCCD, and no protein is removed from the particles in the extraction procedure. Investigation of the binding of ^{113}Sn Triethyl tin shows that there is no difference in the binding of the inhibitor to the high affinity site (Fig. 3.1). There is a 30% decrease in the binding of the triethyl tin at higher concentrations

TABLE 3.1. Extraction of Beef Heart submitochondrial particles

Solvent	ATPase μ mole/min/mg	
	+ oligomycin	- oligomycin
-	0.19	2.15
Diethyl ether	2.03	2.19
Methyl acetate	2.06	1.98
Carbon tetrachloride	1.80	1.76

ATPase assays were performed according to Wakabayashi and Gunge (163), solvent extraction was performed as described by Broughall (159).

TABLE 3.2. Insensitivity of ether extracted Y_{ETP} to various inhibitors

Inhibitor and concentration	ATPase $\mu\text{mole min}^{-1} \text{mg}^{-1} \text{protein}$	
	Control Y_{ETP}	$\text{Et}_2\text{O-}Y_{ETP}$
-	1.10	1.15
oligomycin $1\mu\text{g/mg}$	0.65	0.90
rutamycin $1\mu\text{g/mg}$	0.54	1.05
venturicidin $1\mu\text{g/mg}$	0.36	1.00

ATPase activity was measured by the method of Pullman (146). Ether extraction was performed as described by Broughall (159).

TABLE 3.3. Ether extraction of ETP₁₁ and removal of inhibitor sensitivity

Inhibitor and Concentration	ATPase $\mu\text{moles min}^{-1} \text{mg}^{-1} \text{protein}$	
	Control ETP	Ether ETP
A -	1.32	1.03
oligomycin 16.6 $\mu\text{g}/\text{mg}$	0.15	0.93
DCCD 2nmoles/mg	0.15	1.32
DCCD 2nmoles/mg + oligomycin 16.6 $\mu\text{g}/\text{mg}$	-	1.37
B -	1.32	0.58
rutamycin 16.6 $\mu\text{g}/\text{mg}$	0.34	0.49
ossamycin 16.6 $\mu\text{g}/\text{mg}$	0.25	0.44
peliomycin 16.6 $\mu\text{g}/\text{mg}$	0.22	0.49
venturicidin 16.6 $\mu\text{g}/\text{mg}$	0.15	0.74
C -	0.83	0.69
Triethyl tin 10nmoles/mg	0.09	0.83
D -	0.98	0.58
Aurovertin B 16.6 $\mu\text{g}/\text{mg}$	0.49	0.27
E -	0.63	0.54
NCCD 6nmoles/mg	0.38	0.49

ATPase activity was measured by the method of Pullman (146). Ether extraction was performed as described by Broughall (159).

ATPase activity in Ether-ETP is cold labile, hence the specific activity in B is lower than the control. Similarly, the apparent stimulation in B by venturicidin, and by triethyl tin in C, is due to these assays being performed before the control.

TABLE 3.4. Insensitivity of ether extracted ATPase to various inhibitors

Inhibitor and concentration	ATPase $\mu\text{mole min}^{-1} \text{mg}^{-1} \text{protein}$	
	Control SMP	$\text{Et}_2\text{O-SMP}$
-	1.19	1.13
ossamycin 16.4 $\mu\text{g}/\text{mg}$	0.24	1.06
oligomycin 16.4 $\mu\text{g}/\text{mg}$	0.32	1.03
peliomycin 16.4 $\mu\text{g}/\text{mg}$	0.32	1.16
venturicidin 16.4 $\mu\text{g}/\text{mg}$	0.42	1.24
triethyl tin 16.4nmoles/mg	0.42	1.16

ATPase activity was measured by the method of Pullman (146). S. barbata flight muscle SMP were extracted with ether at 0°C according to Broughall (159).

TABLE 3.5. Reactivation of DCCD inhibited ATPase

	ATPase $\mu\text{moles min}^{-1} \text{mg}^{-1} \text{protein}$	
	- oligomycin	+ oligomycin
Submitochondrial particles (SMP)	1.19	0.32
DCCD-SMP	0.40	-
SMP(Et_2O)	1.13	1.03
DCCD-SMP(Et_2O)	1.16	-

Submitochondrial particles from S. barbata flight muscle were preincubated with DCCD at 1nmole DCCD/mg protein at 0°C for 18 hours (DCCD-SMP). Ether extraction was performed according to Broughall (159) to give SMP(Et_2O). ATPase assays were performed by the method of Pullman (146).

TABLE 3.6. Binding of ^{14}C DCCD to $\text{ETP}_\text{H}(\text{Et}_2\text{O})$

Submitochondrial Particles	% ^{14}C bound
ETP_H	78
$\text{ETP}_\text{H}(\text{Et}_2\text{O})$	75

^{14}C DCCD was incubated with 20mg mitochondrial particles at 1nmole/mg at 0°C for 18 hours. The particles were washed three times in 50ml 250mM Sucrose 10mM Tris-acetate pH 7.5. Radioactivity was measured by liquid scintillation in Triton/toluene/butyl PBD. Ether extraction was performed as described by Broughall (159).

Fig 3.1
EFFECT OF DIETHYL ETHER ON ^{113}Sn TET BINDING IN YETP.

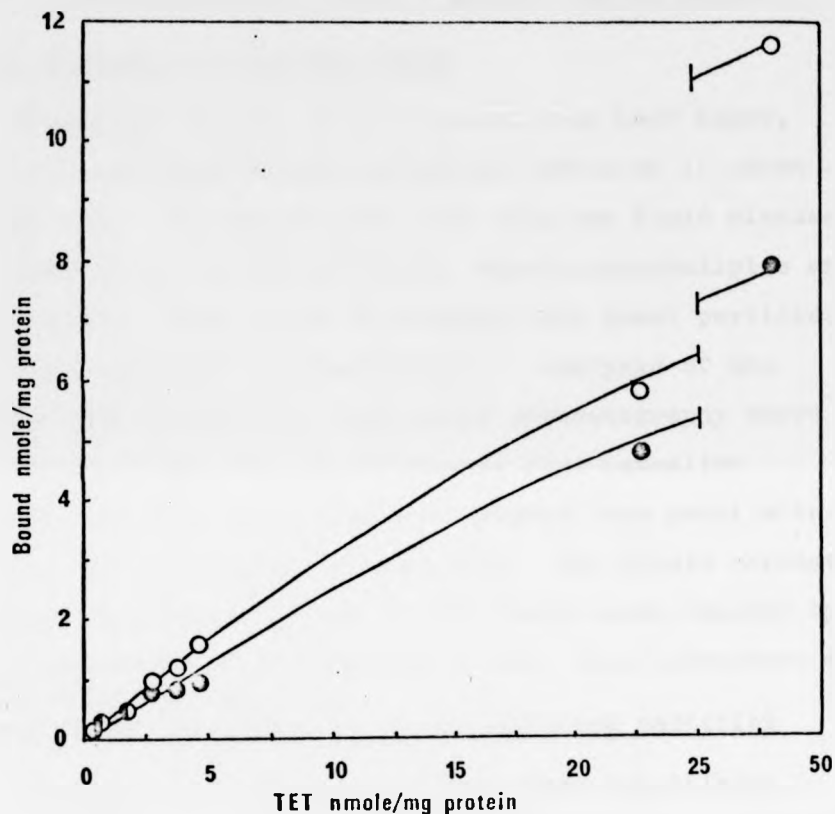


FIGURE 3.1.

Yeast submitochondrial particles (2mg) were incubated at 0°C with varying amounts of ^{113}Sn triethyl tin in 5ml 250mM Sucrose, 10mM Tris-acetate pH 7.5. After 30 minutes, the suspension was centrifuged and the pellet dissolved in 1ml 2% triton X-100. Radioactivity was measured in a Packard Tricarb scintillation counter.

Y_{ETP} ○—○

$Y_{\text{ETP}}(\text{Et}_2\text{O})$ ●—●

although this is not found at the lowest concentrations , indicating a modification or extraction of the low affinity site.

Lipids extracted by diethyl ether

Separation of the ether extracts from beef heart, rat liver and yeast submitochondrial particles is shown in Fig. 3.2. It can be seen that only two lipid classes are found in all of the extracts, namely phospholipids and fatty acids. More lipid is removed from yeast particles than from rat liver and beef heart. Analysis of the phospholipid fraction by thin layer chromatography shows that phosphatidyl choline is removed from mammalian mitochondria, but cardiolipin is removed from yeast mitochondria as illustrated in Fig. 3.3. Gas liquid chromatography of the methyl esters of the fatty acids removed by ether extraction is illustrated in Fig. 3.4. Hydrocarbons are also extracted by diethyl ether.

Properties of the ATPase of ether extracted particles

In addition to the loss of inhibitor sensitivity, other properties of the membrane bound ATPase are altered by extraction with organic solvents. Extraction with methyl acetate at concentrations over 25% leads to total loss of activity. Extraction with all of the organic solvents used leads to cold lability of the ATPase. No decrease in activity is found with diethyl ether. The pH profile of ATPase in normal and ether extracted particles is shown in Fig. 3.5. It can be seen that no relative change in activity occurs at either the pH 9.5 or pH 6.5 maxima.

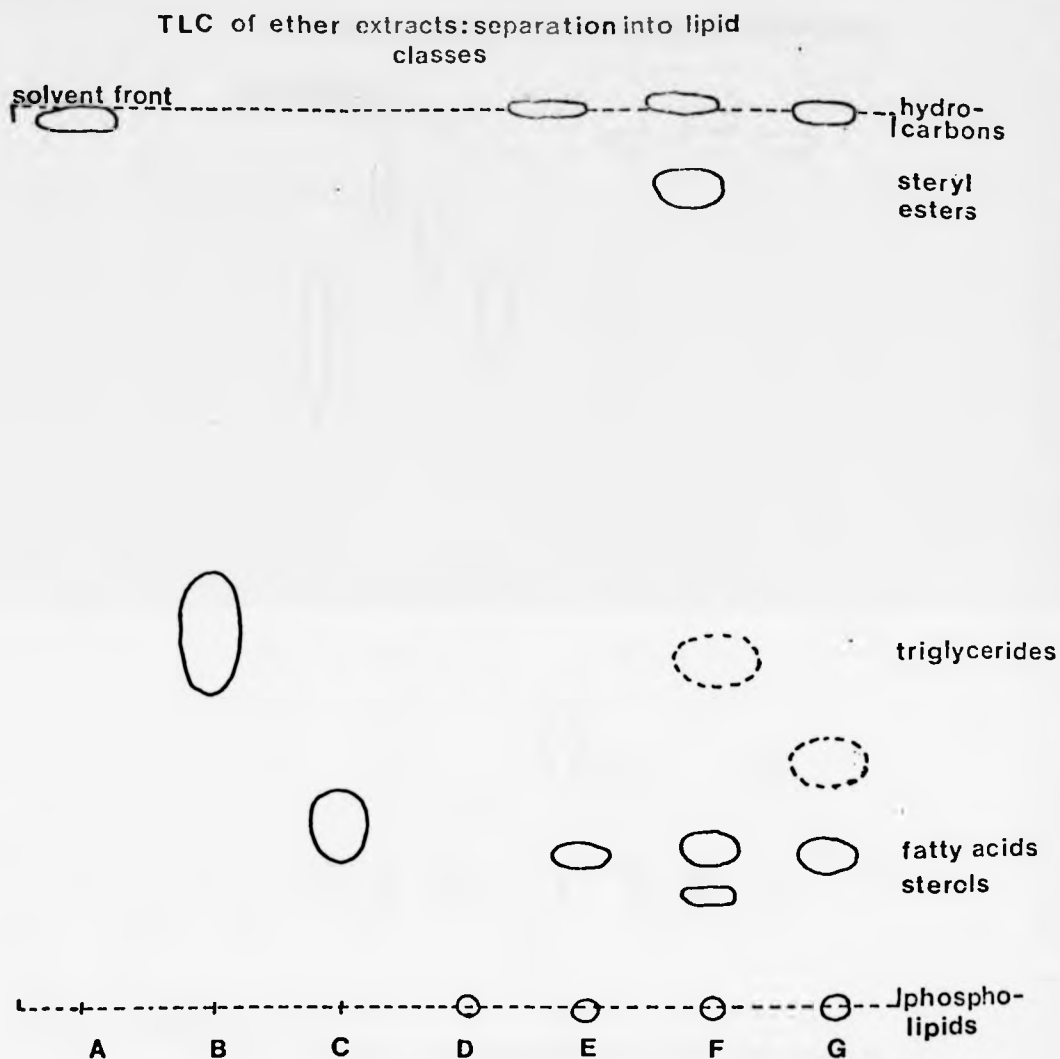


FIGURE 3.2.

TLC was performed on 0.5mm Silica Gel plates. Solvent was petroleum ether/diethyl ether/acetic acid (90:10:1 v/v). Spots were visualised with iodine vapour. Dashed spots are very faint.

A - tetradecane B - triolein C - palmitic acid
 D - lecithin E - Ether extract from beef heart SMP
 F - Ether extract from yeast SMP
 G - Ether extract from rat liver SMP

TLC of ether extracts: phospholipid separation

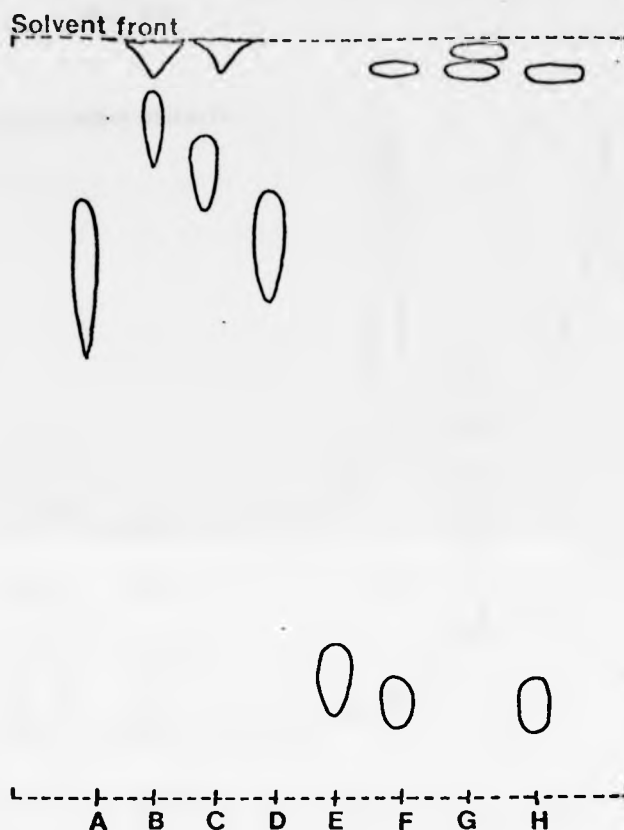


FIGURE 3.3.

TLC was performed on Silica Gel N (+ 120mg Ammonium sulphate/30g) 0.5mm plates. Solvent was Chloroform/Methanol/Acetic acid/water (50:25:8:1 v/v). Spots were visualised with iodine vapour. A - phosphatidyl serine, B - diphosphatidylglycerol, C - phosphatidic acid, D - phosphatidylethanolamine, E - phosphatidylcholine, F - Ether extract from beef heart SMP, G - Ether extract from yeast SMP, H - Ether extract from rat liver SMP.

Fig 3.4.

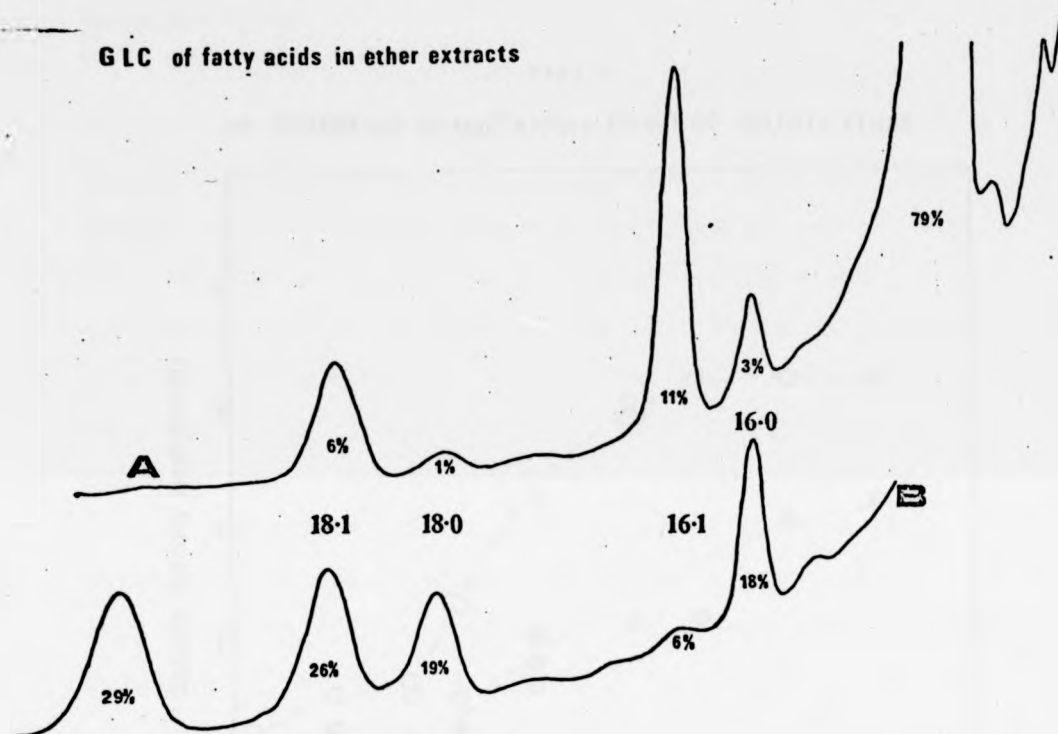


FIGURE 3.4.

Fatty acid composition of ether extracts of Y_{ETP} and ETP_H . Fatty acids are expressed as a percentage of the total fatty acids. The fatty acids are denoted by the convention number of carbon atoms: number of double bonds. Fatty acids were determined as their methyl esters as described by Watson *et al.* (186).

A - yeast mitochondria, B - bovine heart mitochondria

Fig 3.5

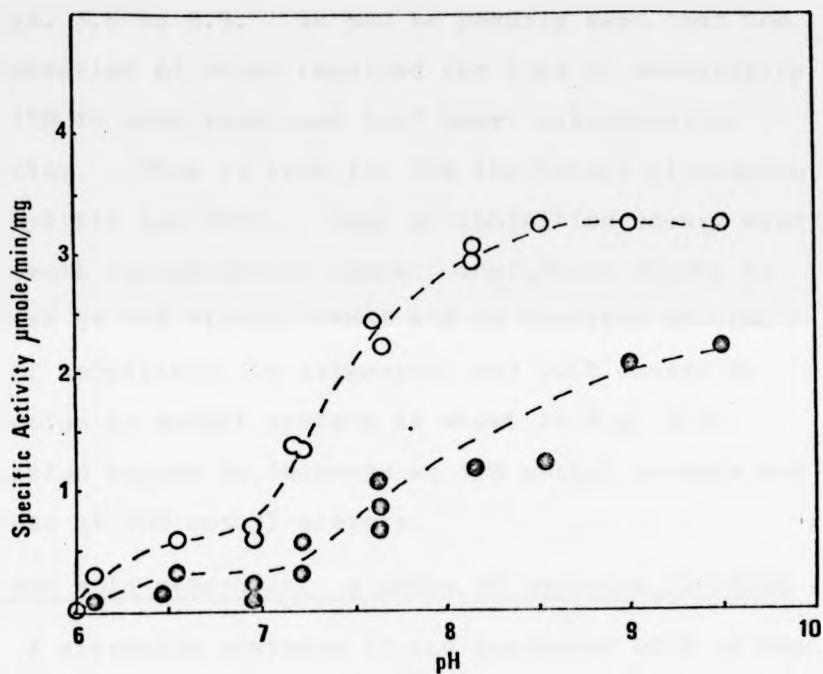
pH DEPENDENCE OF $Mg^{2+}ATPase$: EFFECT OF DIETHYL ETHER

FIGURE 3.5.

ATPase activity was measured by the method of Wakabayashi and Gunge (163). Ether extraction was performed essentially as described by Broughall (159) without evaporation of ether with nitrogen. \bigcirc — \bigcirc Y_{ETP} , \bullet — \bullet $Y_{ETP} (Et_2O)$.

The critical concentration of solvent required for loss of sensitivity

Titration of the concentration of diethyl ether required for loss of inhibitor sensitivity is illustrated in Figs. 3.6 to 3.9. It can be readily seen that the concentration of ether required for loss of sensitivity is 12 - 15% in both yeast and beef heart mitochondrial particles. This is true for the inhibitors oligomycin, triethyl tin and DCCD. Loss of inhibition occurs over a very small concentration range: inhibition starts to decrease at 10% diethyl ether and is complete at 15%. Loss of sensitivity to oligomycin and DCCD caused by extraction by methyl acetate is shown in Fig. 3.9. Inhibition begins to decrease at 15% methyl acetate and is complete at 20% methyl acetate.

Electron spin resonance: a probe of membrane fluidity

A nitroxide analogue of the inhibitor DCCD is the compound NCCD. The respective structures of NCCD and DCCD are shown in Fig. 3.10. The degree of inhibition of ATPase in beef heart submitochondrial particles is very similar as shown in Fig. 3.11. The E.S.R. spectrum of NCCD is shown in Fig. 3.12. Incubation of the spin probe with beef heart submitochondrial particles causes a strong degree of immobilisation as shown by the loss of structure in the spectrum. The degree of immobilisation is so great that it is impossible to determine any of the parameters from the E.S.R. spectrum. Extraction with diethyl ether does not change the mobility of the probe

Fig 3.6

EFFECT OF DIETHYL ETHER ON INHIBITOR SENSITIVITY IN YETP

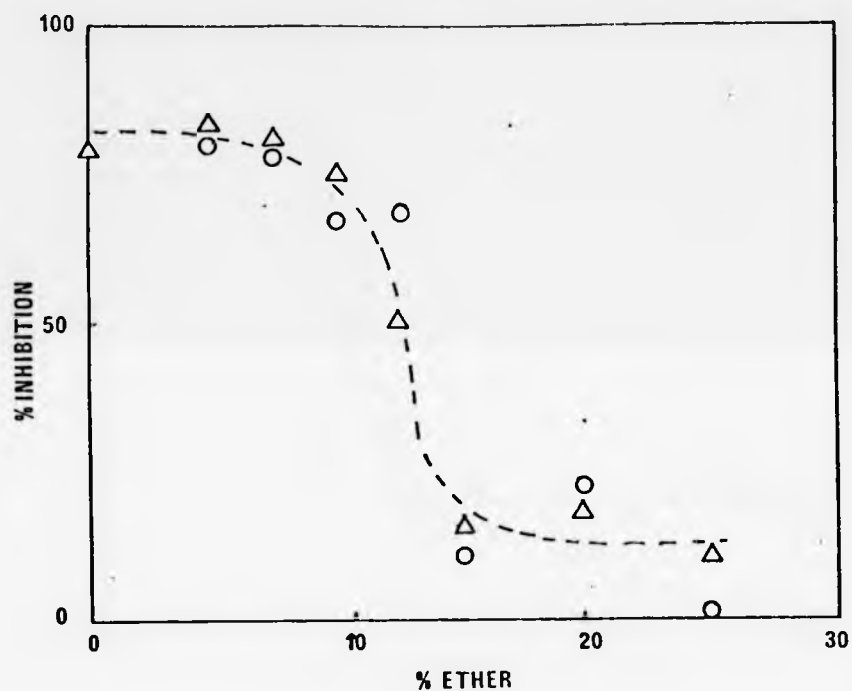


FIGURE 3.6.

ATPase activity was measured by the method of Wakabayashi and Gunge (163). Ether extraction was performed essentially as described by Broughall (159) without evaporation of ether with nitrogen.

○—○ Y_{ETP} + triethyl tin
 △—△ Y_{ETP} + oligomycin

Fig 3.7

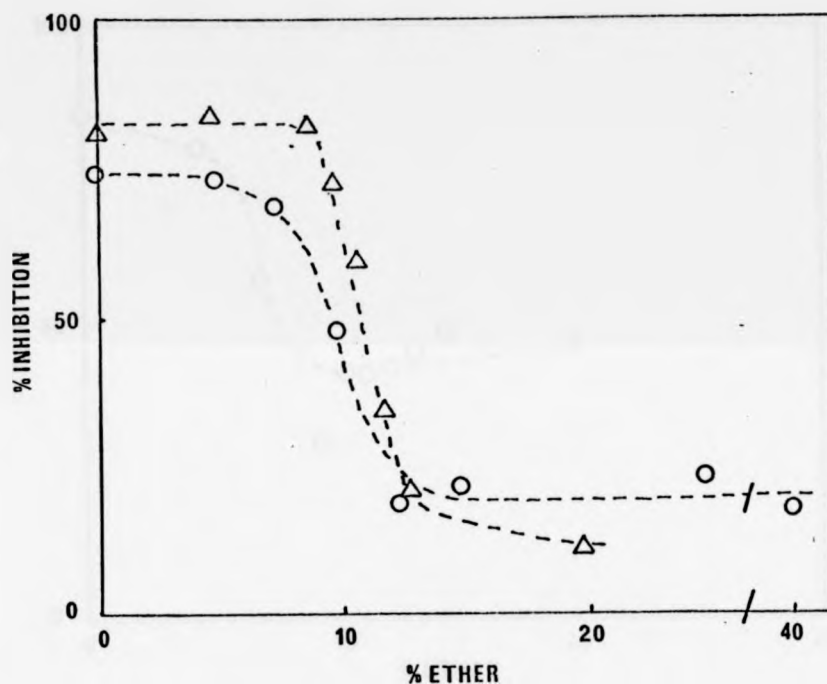
EFFECT OF DIETHYL ETHER ON INHIBITOR SENSITIVITY IN ETP_H 

FIGURE 3.7.

ATPase activity was measured by the method of Wakabayashi and Gunge (163). Ether extraction was performed essentially as described by Broughall (159) without evaporation of ether with nitrogen.

○—○ ETP_H + triethyl tin
 △—△ ETP_H + oligomycin

Fig 3.8

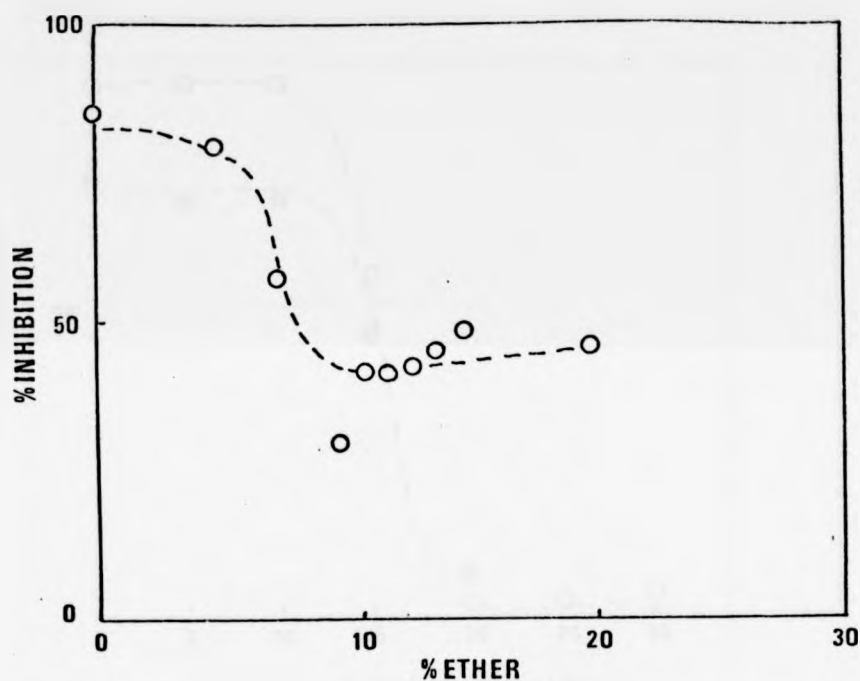
EFFECT OF DIETHYL ETHER ON DCCD SENSITIVITY IN ETP_H 

FIGURE 3.8.

ATPase activity was measured by the method of Wakabayashi and Gunge (163). Ether extraction was essentially performed as described by Broughall (159). DCCD was incubated with ETP_H for 18 hours at 0°C , prior to extraction with diethyl ether.

Fig 3.9

EFFECT OF METHYL ACETATE ON INHIBITOR SENSITIVITY

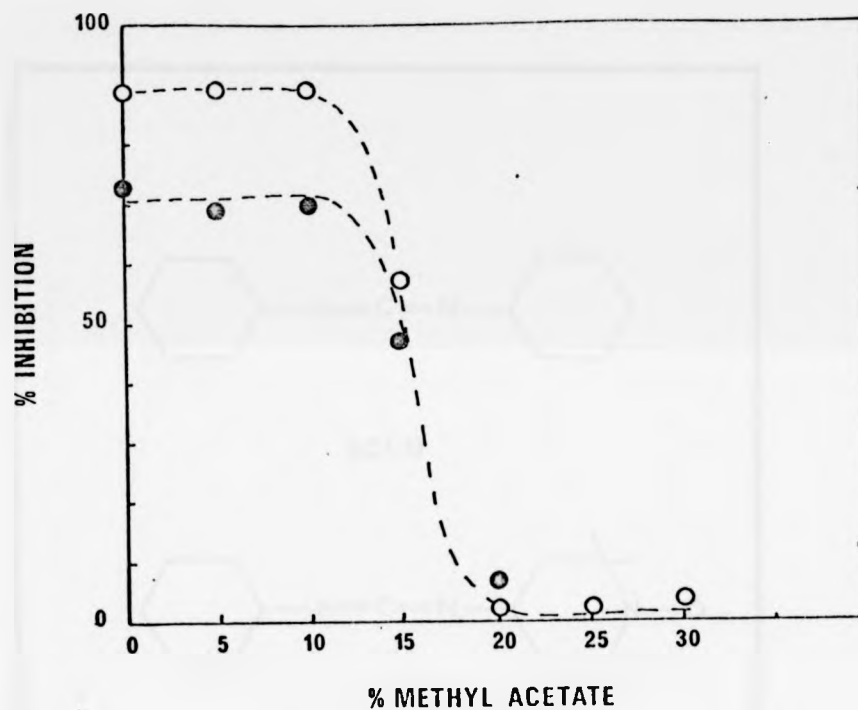
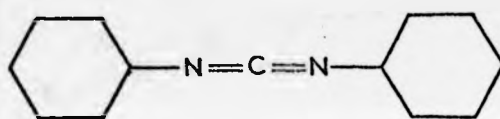


FIGURE 3.9.

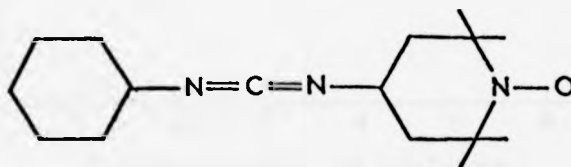
ATPase activity was measured by the method of Wakabayashi and Gunge (163). Extraction of Y_{ETP} with methyl acetate was performed at 0°C.

- Y_{ETP} + oligomycin
- Y_{ETP} + triethyl tin.

Fig 3.10



DCCD



NCCD

Fig 3.11

Inhibition of heart mitochondrial ATPase by DCCD
and NCCD

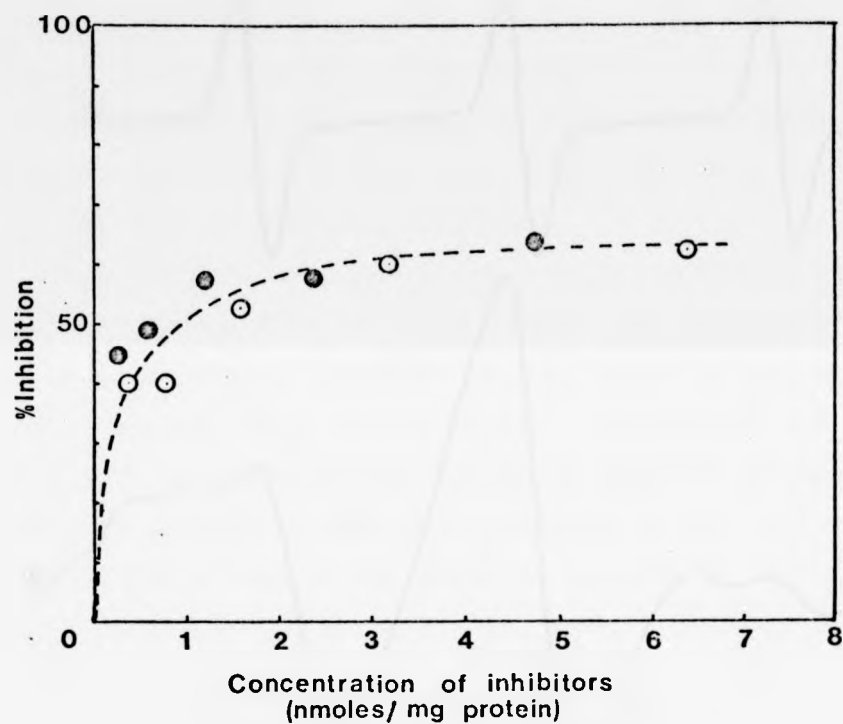


FIGURE 3.11

ATPase assays were performed according to Wakabayashi and Gunge (163). Inhibitors were preincubated with ETP_H at 0°C for 18 hours.

○—○ DCCD ●—● NCCD.

Fig 3.12

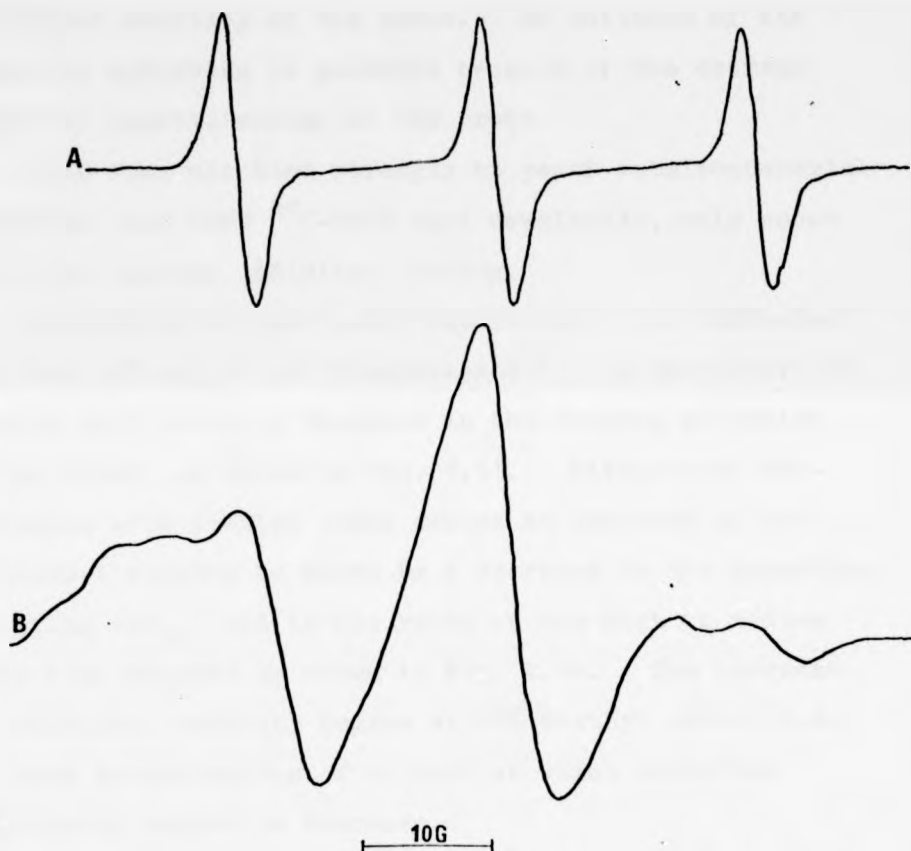


FIGURE 3.12.

A. ESR spectrum of NCCD in 250mM
 Sucrose 10mM Tris-acetate pH 7.5.

B. ESR spectrum of NCCD in beef heart mitochondrial
 membranes.

NCCD was incubated with ETP_H at 1nmole/mg mitochondrial
 protein at 0°C for 18 hours. Free NCCD was removed
 by repeated washing with sucrose-tris.

as can be seen in Table 3.7. The ratio of the high to medium field line heights does not alter significantly over the range 0-50% ether, indicating no change in the rotational mobility of the probe. No estimate of the hyperfine splitting is possible because of the extreme degree of immobilisation of the probe.

NCCD does not bind strongly to yeast submitochondrial particles, nor does ^{14}C -DCCD bind covalently, only about 3% of the applied inhibitor binding.

Incubation of beef heart submitochondrial particles with the 5-N-oxyl-4'-4'-dimethyloxazolidine derivative of stearic acid causes a decrease in the freedom of motion of the label, as shown in Fig. 3.13. Titration of the particles with diethyl ether causes an increase in the rotational freedom as shown by a decrease in the hyperfine splitting ($2T_{11}$) and in the ratio of the high to medium field line heights as shown in Fig. 2.14. The increase in rotational mobility begins at 10% diethyl ether, i.e. the same concentration of solvent at which inhibitor sensitivity begins to decrease.

3.4. Discussion

Extraction of beef heart mitochondrial particles with a variety of organic solvents results in the loss of inhibitor sensitivity. Diethyl ether was chosen as the most suitable solvent for a variety of reasons; (a) diethyl ether is relatively easily removed, (b) no loss of ATPase activity is found on extraction with diethyl ether, although the ATPase is rendered cold labile as found with

TABLE 3.7. Effect of diethyl ether on the mobility of
NCCD

% Ether	A/B
0	0.74
5	0.72
20	0.74
50	0.74

NCCD was incubated with ETP_H at 1nmole/mg protein for 18 hours at 0°C as described by Azzi (179). Diethyl ether was added to the concentrations shown and shaken on a rotary mixer. ESR spectra were recorded on a Decca Radar X1 with a Newport Instruments 7 inch magnet.

Fig 3.13

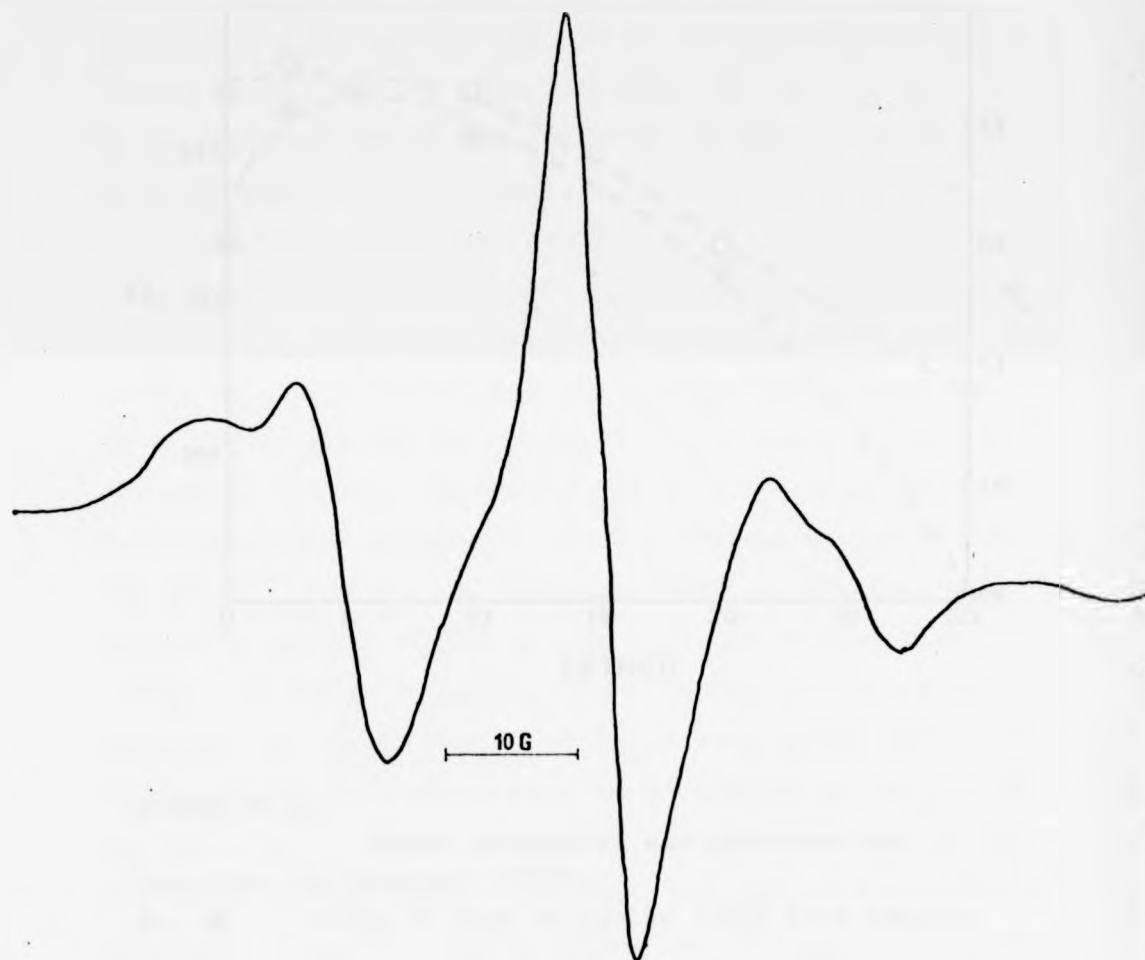


FIGURE 3.13.

ESR spectrum of the 5-N-oxyl 4',4' dimethyloxazolidene derivative of stearic acid incorporated into beef heart submitochondrial membranes. For methods see text.

Fig 3.14

EFFECT OF DIETHYL ETHER ON MOBILITY OF STEARIC ACID

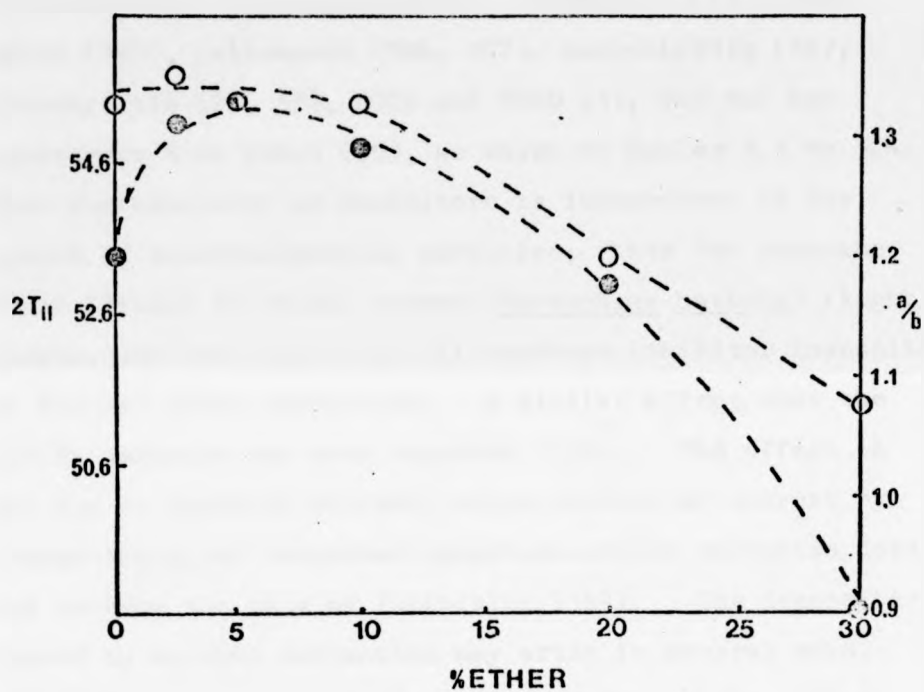


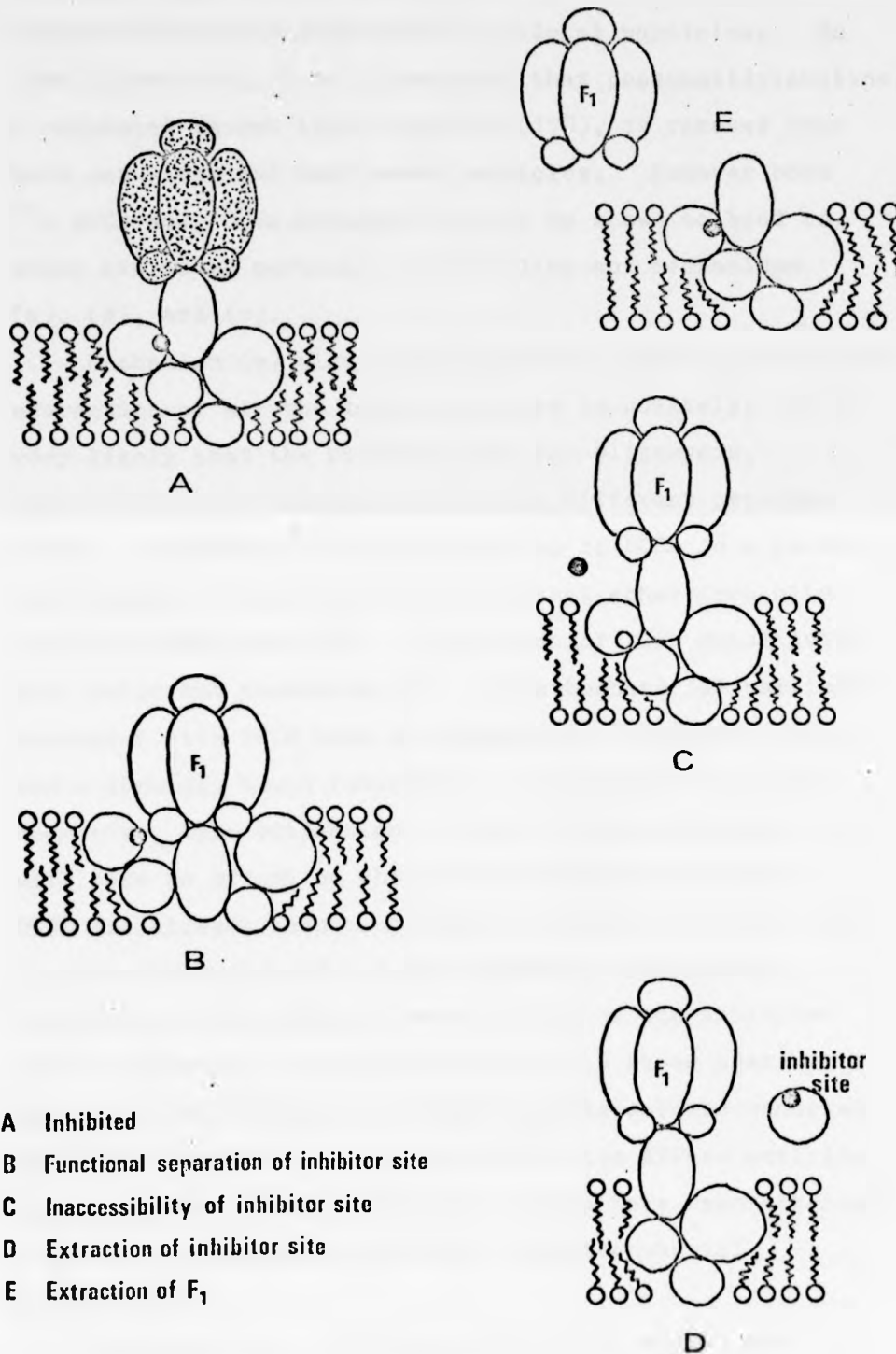
FIGURE 3.14.

Ether extraction was performed as described by Broughall (159).

- ⊗--⊗ ratio of high to medium field line heights
- $2T_{II}$

other solvents, (c) it is known that diethyl ether removes no protein and relatively little lipid from mitochondrial particles (157). Diethyl ether extraction confers insensitivity to oligomycin (9), rutamycin (29), ossamycin (167), peliomycin (166, 167), venturicidin (34), triethyl tin (29, 33), DCCD and NCCD (31, 32) but not aurovertin B or Dio-9 (35), as shown by Tables 3.1 to 3.4. This insensitivity to inhibitors is independent of the source of submitochondrial particles; thus the membrane bound ATPases of yeast, insect (Sarconhara barbata) flight muscle, and beef heart are all rendered inhibitor insensitive by diethyl ether extraction. A similar effect upon the ATP/Pi exchange has been reported (168). The effect is not due to residual solvent, since removal of solvent by freeze-drying of extracted submitochondrial particles does not reverse the loss of inhibition (169). The insensitivity caused by solvent extraction may arise in several ways, of which the most likely are shown in Fig. 3.15; (a) a target lipid may be removed which is responsible for transport of the inhibitor to the binding site, (b) there may be a conformational change in the ATPase such that the inhibitor binding peptide does not interact with the ATPase catalytic site, (c) the F_1 ATPase may be removed, (d) the binding site may be made inaccessible to the inhibitor, (e) the inhibitor binding site may be extracted. Mechanism (a) seems improbable considering the variety of inhibitors and particles in which the effect can be found. Examination of the ether extracts by TLC and GLC, as shown in Figs. 3.2 to 3.4, shows no similarities between

SOLVENT EXTRACTION
POSSIBLE MECHANISMS FOR LOSS OF INHIBITOR SENSITIVITY



extracts from different submitochondrial particles. In this connection, it is interesting that phosphatidylcholine, a suggested target lipid for DCCD (170), is removed from both rat liver and beef heart particles. However both ^{14}C DCCD and ^{113}Sn Triethyl tin can be shown to bind to ether extracted particles, thus ruling out mechanisms (a), (d), and (e).

Mechanism (e) also seems improbable since simultaneous extraction of all the inhibitor sites is unlikely; it is very likely that the binding sites for oligomycin, venturicidin and triethyl tin lie on different peptides (114). Moreover, the site of action of DCCD is a proteo-lipid which is not extracted by diethyl ether from mitochondrial membranes (78). Induction of DCCD sensitivity also rules out mechanism (d). Submitochondrial particles incubated with DCCD have an irreversibly inhibited ATPase and a strongly bound inhibitor. If mechanism (d) is operative, then extraction of the DCCD-treated particles will have no effect on the inhibited ATPase since the DCCD has already been bound to the inhibitor binding site. If mechanisms (b) and (c) are operative then solvent extraction will lead to a reactivation of the inhibited ATPase activity. The data in Table 3.5 shows that extraction of DCCD treated flight muscle submitochondrial particles results in a restoration of the ATPase activity to the control level. Similar results have been obtained with DCCD and NCCD in beef heart submitochondrial particles.

Mechanism (c), the extraction of F_1 , would seem

likely from some of the properties of the ether treated ATPase: cold lability, insensitivity to oligomycin, sensitivity to Dio-9 and aurovertin B. The extraction of the catalytic site is probably not the case for diethyl ether extraction, although extraction with chloroform does operate in this way (171). No protein is found in the supernatant after diethyl ether extraction (169). No ATPase activity is to be found in the supernatant after diethyl ether extraction, nor is there a decrease in specific activity of the extracted particles, other than that due to the cold lability of the ATPase (169). This would appear to rule out mechanism (c).

Mechanism (b), the functional separation of the inhibitor site from the ATPase, has been suggested by Lenaz (155) as the mode of action of short chain alcohols on beef heart mitochondrial ATPase. The concentration of alcohol required for extraction of phospholipid is much higher than that required for loss of oligomycin sensitivity (154) and it is suggested that low concentrations of alcohols disrupt the lipid bilayer and thus alter the conformation of the ATPase complex (155). Lenaz has noted a correlation between the effectiveness of the alcohols and their chain length (154). A similar effect has been found by Hegyvary (172); the Na^+/K^+ ATPase of the plasma membrane is inhibited by alcohols. This can be shown to be due to an increased fluidity of the membrane and a disruption of hydrophobic interactions. The concentration of alcohols giving half maximal inhibition of the Na^+/K^+ ATPase may be correlated with their chain length or

the octanol-water partition coefficients (172). Use of a nitroxide labelled sterol shows that the hyperfine splitting constant decreases at the same concentration which causes half maximal inhibition (173). Lenaz has shown that n-butanol increases the fluidity of the beef heart mitochondrial membrane as measured by the E.S.R. spectrum of the 5-, 12-, and 16-N-oxyl 4',4' dimethyl-oxazolidine derivatives of stearic acid (154). Similarly, the half maximal loss of inhibition induced by diethyl ether occurs at about 12% diethyl ether. This critical concentration of diethyl ether appears to be the same for oligomycin, triethyl tin and DCCD. It is independent of the source of the submitochondrial particles. Examination of the E.S.R. spectrum of the 5-N-oxyl 4',4' dimethyl-oxazolidine derivative of stearic acid shows that the fluidity of the membrane increases at this same concentration of diethyl ether. It would seem likely therefore that the functional separation of the inhibitor site from the ATPase arises from a modification of protein-lipid interactions. The hydrophobic interaction of membrane proteins with lipids may be extensively modified by an increase in the fluidity of the lipid bilayer. This is supported by the observations of Lenaz (154) that the activation energy of beef heart mitochondrial ATPase is increased three fold by n-butanol, although no data is given on the displacement of the break point in Arrhenius plots of the ATPase. Also, the ratio of the high to middle field line heights of the nitroxide labelled stearic acids decrease to the level found in phospholipid

vesicles (154). This ratio may be taken as an indication of rotational freedom (174).

Prompted by the similarity of the critical concentration of diethyl ether to the solubility of the solvent in water, an investigation of the critical concentration of other solvent shows that the loss of inhibition and the increase in fluidity of the mitochondrial membrane occur at the concentration at which the aqueous phase is saturated with solvent. This has been found for diethyl ether, carbon tetrachloride, and methyl acetate. Kates (175) has shown that phospholipases in plastids are stimulated by diethyl ether, and that maximal stimulation is found at the concentration at which the aqueous phase is saturated with diethyl ether. While the stimulation of phospholipase is not advanced as a possible mechanism for the loss of inhibitor sensitivity in mitochondria, the suggestion (176) that diethyl ether at saturating concentrations causes surface dilution of the closely packed molecules at the lipid-water interface may provide reason for the increase in membrane fluidity. Lenaz (156) has suggested that specific phospholipids are extracted by alcohols and ether, labilising protein-lipid bonds in the membrane.

The conformation of membrane proteins has been shown to be profoundly affected by lipid depletion (153). Cunningham has found that ether treatment prevents the visualisation of inner membrane spheres by negative staining (177). This has been found to be true of other organic solvents which cause loss of inhibitor sensitivity (168).

Similarly Kagawa and Racker (58) and Kopaczyk (59) have shown that in oligomycin sensitive ATPase phospholipids were essential for oligomycin sensitivity and visualisation of inner membrane spheres. Although this would appear to support the notion of a conformational change in the ATPase, interpretation of negatively stained preparations must be made with caution; freeze-etched preparations of the matrix face of the inner mitochondrial membrane do not display projections corresponding to those found by negative staining (178).

Although the change in the ATPase is not sufficient to cause gross changes in the properties of the ATPase activity such as the pH dependence, it was considered possible that a change in conformation of the DCCD binding protein might be monitored by use of the spin label analogue NCCD (179). The nitroxide analogue of DCCD is very similar (Fig. 3.10) in structure, and the inhibition of the ATPase activity by NCCD and DCCD is very similar (Fig. 3.11). The E.S.R. spectrum of NCCD bound to beef heart submitochondrial particles shows that the inhibitor is bound very strongly to the membrane. Either extraction of NCCD treated particles removes the inhibition completely, but this is not due to a change in the mobility of the NCCD as shown by Table 3.7. However, Azzi *et al.* (179) have shown that the distance between the ATP-Mn²⁺ binding site and that of NCCD can be calculated from the interaction between the two spin probes to be approximately 20Å. and that this distance increases to a value higher than 35Å on diethyl ether extraction (180).

CHAPTER 4. AN INVESTIGATION OF THE Mg^{2+} ATPase OF MUTANTS OF *S. CEREVISIAE* WITH LOWERED SENSITIVITY TO OLIGOMYCIN

4.1. Introduction

Classically, elucidation of the mechanism of oxidative phosphorylation has been pursued by the isolation of "coupling factors", presumed to be intermediates in the energy conservation process, and reconstitution of various enzymic activities in depleted particles by these partially purified membrane fractions. The degree of confusion arising from the heterogeneity of some of these factors, and the variety of mechanisms by which reconstitution may operate, leads one to question the value of this approach. Two alternative approaches to this problem are the labelling of specific proteins by inhibitors acting at known sites of oxidative phosphorylation (15, 16), and the genetic manipulation of yeast to produce a modified coupling system (102, 181). Mutants of *Saccharomyces cerevisiae*, resistant to inhibitors of oxidative phosphorylation such as oligomycin, venturicidin and triethyl tin compounds, have been isolated in this laboratory (110, 112, 114). These mutants have been shown to possess a modified coupling system in which both ATP/Pi exchange and ATPase are resistant to the inhibitors (116, 182). Although this lowered sensitivity has been shown to exist in the OS-ATPase complex (116, 183), and that the site of the modification lies in the so called "membrane fraction", no protein of the mitochondrial membrane has so far been shown to have been altered in the mutants. It has been

suggested that the lowered sensitivity of the mutants may arise from a modification of the lipid composition of the OS-ATPase (183), although this would seem unlikely in view of the cytoplasmic nature of the mutations (111, 113). The protein nature of the oligomycin binding site has not been shown in any unequivocal experiment, although the oligomycin and F_1 binding sites appear to be trypsin labile (192). OSCP, once thought to be the site of action of oligomycin, is almost certainly not the (sole) binding site of the inhibitor (71), and is not the component modified in the oligomycin resistant mutants. It was decided, therefore, to investigate the cross resistance of oligomycin resistant strains to DCCD, an inhibitor of oxidative phosphorylation which is thought to act by covalent interaction with a protein of the OS-ATPase complex (32, 78).

4.2. Methods and Materials

Strains and Growth of Yeast

The yeast strain used in this study was D22 (ad_2^+), and the mutants isolated from this parental strain as described by Avner (110, 184). Batch culture was carried out in a New Brunswick Fermentor in 10L of culture medium containing 0.1% peptone, 0.5% yeast extract, 100ml/10L Wickerhams salts (185), 0.05% Adenine sulphate, 1% ethanol and 5ml/10L tributyl citrate as an antifoaming agent. Solid media for cross-resistance experiments was buffered at pH 7.5 with 0.1M phosphate.

Preparation of mitochondria and OS-ATPase

Mitochondria and submitochondrial particles were prepared as described by Watson *et al.* (186). Mitochondria were further purified on a discontinuous sucrose gradient (20 - 70% w/v). Oligomycin sensitive ATPase was prepared according to Tzagoloff (40), with extraction of submitochondrial particles with 0.5% Triton X-100 and centrifugation of the solubilised ATPase on a continuous 5 - 20% sucrose gradient containing 0.01% Triton X-100. Active fractions from sucrose density centrifugation runs were pooled and concentrated using Minicon Macrosolute concentrators.

Enzyme Assays and lipid estimation

ATPase activity was assayed by the method of Wakabayashi and Gunge (163) in a volume of 1ml at 30°C, pH 9.5. Reactions were initiated with 10 μ moles ATP and terminated by the addition of 0.5ml 10% TCA. Phosphate was determined by a modification of the method of Fiske and Subbarow (164) scaled down to a total volume of 3ml. Protein was estimated by the biuret method of Gornall *et al.* (141) in the presence of 1% deoxycholate. Phospholipid phosphorus was determined by the method of Bartlett (194).

Ergosterol was determined by the method of Wood (187). Cytochrome (difference) spectra were obtained on an SP1800 spectrophotometer using sodium dithionite and potassium ferricyanide as reducing and oxidising agents.

Materials

Lipids were obtained from Lipid Products Ltd. DCCD and triethyl tin chloride were obtained from British Drug Houses Ltd. Oligomycin was obtained from Sigma Chemical Co. Ltd. and is a mixture of oligomycin A (15%) and oligomycin B (85%). Venturicidin was a gift from Dr. I.D. Fleming (Glaxo Laboratories, Stoke Poges, UK.). All other chemicals were analytical grade.

4.3. Results

DCCD is known to be an inhibitor of oxidative phosphorylation (32), and the toxicity of the inhibitor to the yeast Saccharomyces cerevisiae at the whole cell level can be shown to be due to such inhibition by Figs. 4.1 to 4.3. Growth of the auxotroph D22 on non-fermentable substrate is totally inhibited by DCCD at the low concentration of 5 µg/ml as shown in Fig. 4.1. Growth of D22 on fermentable substrate, however, is inhibited by DCCD only when the organism has adapted to a non-fermentable mode of growth as shown in Fig. 4.2. In addition the cytochrome spectra of yeast grown in the presence of DCCD show loss of cytochromes a and b, a characteristic result of growth in the presence of inhibitors of aerobic metabolism and restriction to fermentative growth (188).

The cross-resistances of a series of oligomycin resistant mutants of D22 isolated in this laboratory are given in Table 4.1. There appears to be no correlation between the oligomycin and DCCD resistances of the OL1

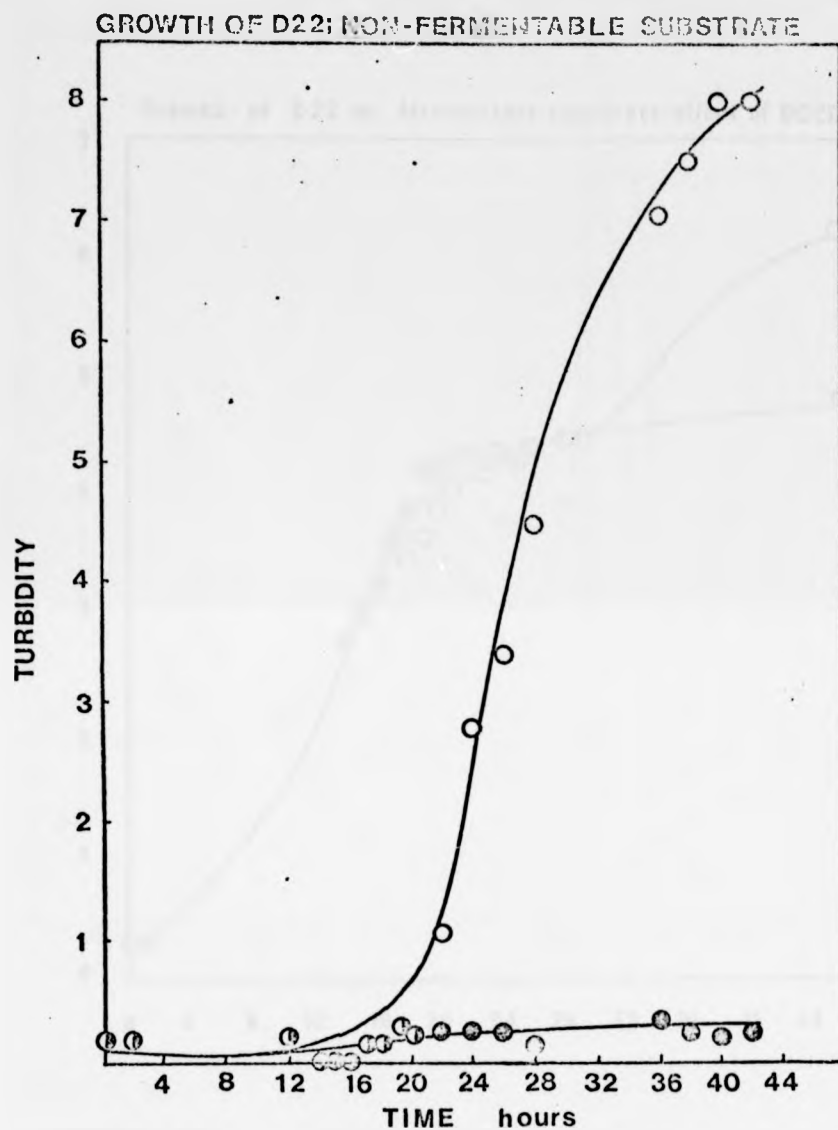


FIGURE 4.1.

1ml inocula from a culture of *S. cerevisiae* D22 was transferred to 50ml sterile culture medium (184) with 1% ethanol as substrate. Growth was monitored by turbidity in an EEL colorimeter (filter 607).

○—○ No DCCD ●—● DCCD at 5µg/ml.

Fig 4.2.

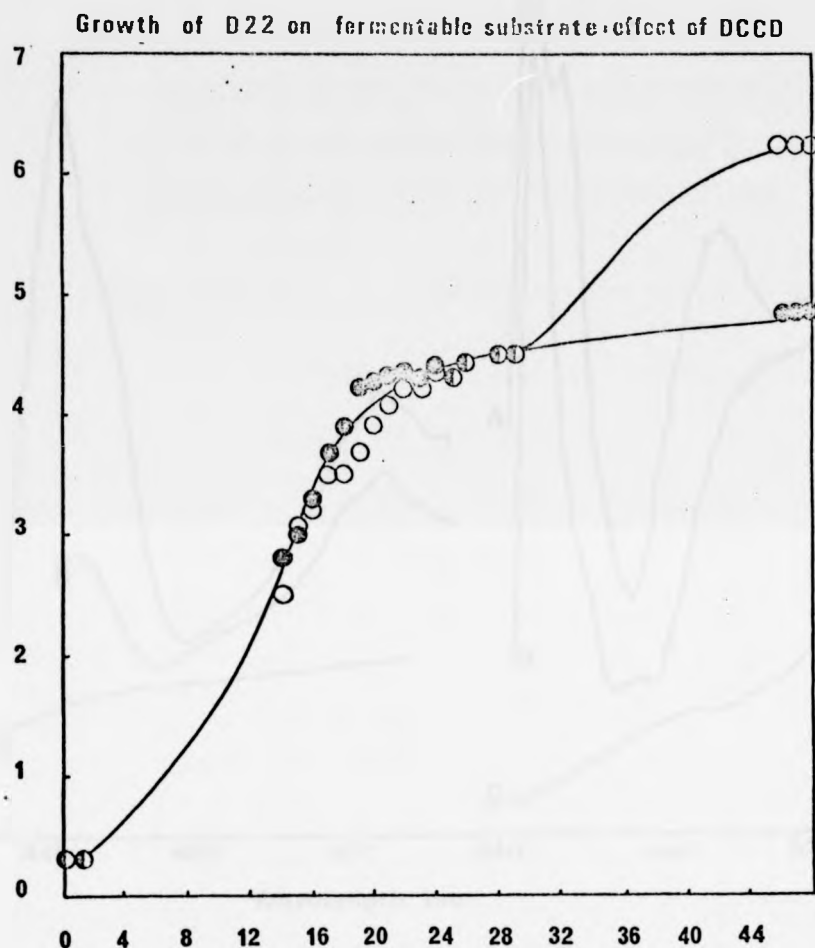


FIGURE 4.2.

1ml inocula from a culture of S. cerevisiae D22 were transferred to 50ml sterile culture medium (184) with 1% glucose as substrate. Growth was monitored by turbidity in an EEL colorimeter (filter 607)

○—○ No DCCD ●—● DCCD at 5µg/ml.

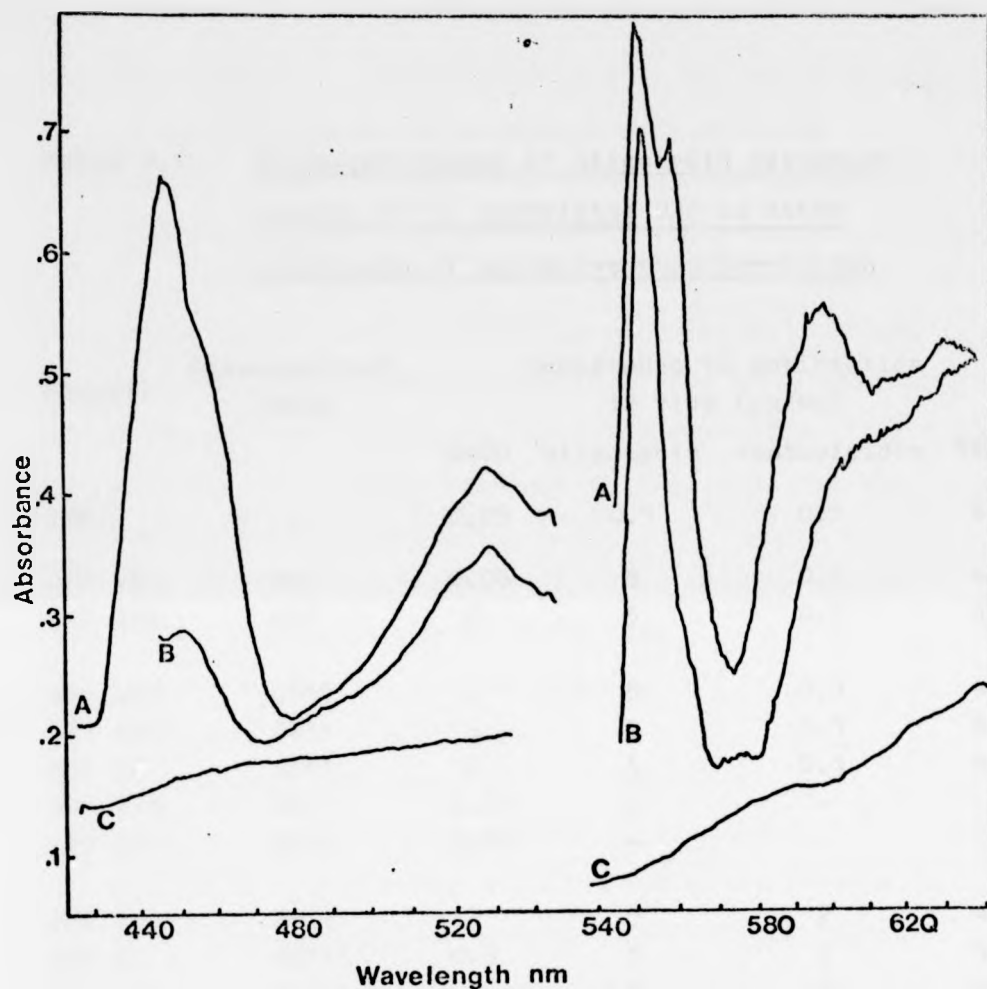


FIGURE 4.3.

Cytochrome difference spectra were recorded on an SP1800 spectrophotometer. Cells were suspended in 50% glycerol at 20mg/ml, sodium dithionite and potassium ferricyanide were used as reducing and oxidising agents.

A. Cells harvested at second stationary phase during growth on 2% glucose.

B. Cells grown as in A but in the presence of 5 μ g/ml DCCD

C. Blank (reduced vs reduced).

TABLE 4.1. Cross-resistance of oligomycin resistant mutants of *S. cerevisiae* D22 to other inhibitors of oxidative phosphorylation

Strain	Mitochondrial locus	Resistance to antibiotics in vivo ($\mu\text{g}/\text{mg}$)			
		DCCD	oligomycin	venturicidin	TET
D22	-	0.05	0.5	0.1	4
D22 A21	OL1	0.05	5	0.1	4
D22 A16	OL1	2	5	0.1	4
D22 A14	OL11	2	5	0.1	4
D22 A15	OL11	5	5	0.1	4
D22 C4	OL11	2	5	0.1	4
D22 A13	OL11	0.05	-	-	-
D22 B21	OL11	0.05	-	-	-
D22 A19	OL111	2	5	2	4
D22 61	OL111	0.5	5	2	4
D22 62	OL111	2	5	2	4
D22 69	V1	2	0.5	-	20

Strains were assayed for resistance to inhibition of growth using the drop-out procedure. The resistance level is defined as the maximum concentration allowing normal growth of the strain. Glycerol medium was used, buffered at pH 7.5 for DCCD, 6.25 for other inhibitors.

and OL11 classes of mutants, but all of the OL111 mutants so far tested display resistance to both DCCD and oligomycin. These mutants are also interesting in that they possess a lowered sensitivity to the inhibitor venturicidin. The lowered sensitivity of the OL111 mutants is maintained at the mitochondrial level; the Mg^{2+} ATPase of submitochondrial particles derived from OL111 mutants has a lowered sensitivity to the inhibitors DCCD (Fig. 4.4), venturicidin (Fig. 4.5), oligomycin (Fig. 4.6) and the aglycone of venturicidin (Fig. 4.7).

The resistance phenomenon in these mutants may be accounted for by a number of mechanisms

- (a) Detoxification in the cytoplasm
- (b) Mitochondrial detoxification
- (c) Permeability barriers to the inhibitors
- (d) Modification of the inhibitor binding site.

Mechanisms (a) and (c) may be ruled out as resistance is maintained in "inside-out" submitochondrial particles. In order to distinguish between mechanisms (b) and (d), and to select those mutants in which the inhibitor binding site is modified, the oligomycin sensitive ATPase was prepared from the parental strain and the mutants.

Surprisingly, since the use of the purified OS-ATPase of Tzagoloff (40) has been extensively reported in the literature, little seems to be known about the kinetics of ATP hydrolysis of this enzyme. A typical preparation of OS-ATPase from D22 is shown in Fig. 4.8. It has been shown that OS-ATPase preparations may require lipids for maximal activity (64), and consequently OS-ATPase purified

Fig 4.4

DCCD Inhibition of Mg ATPase in SMP: OLIII.

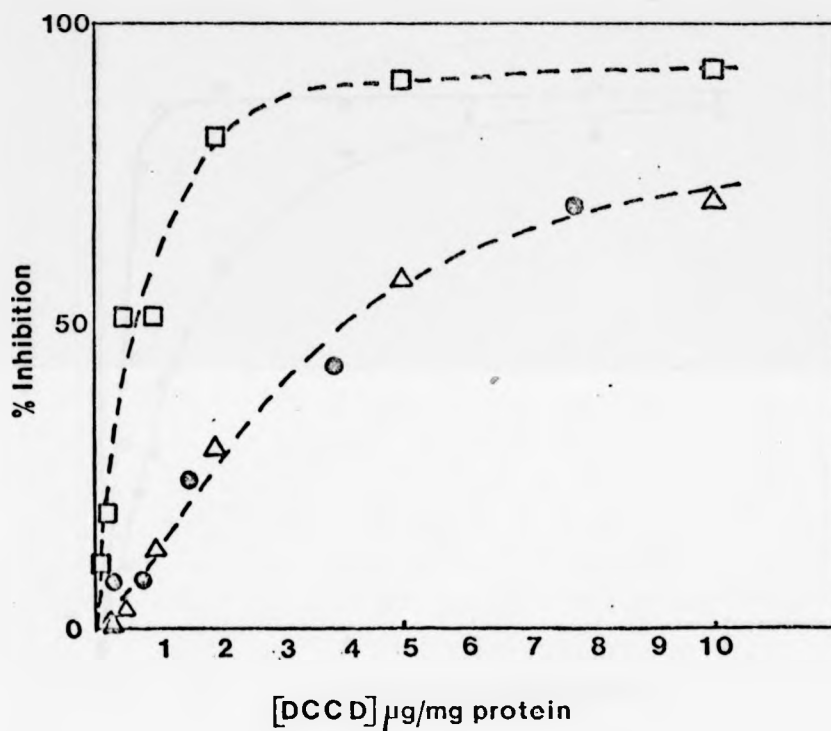


FIGURE 4.4.

ATPase assays were performed by the method of Wakabayashi and Gunge (163). Submitochondrial particles were preincubated with DCCD at varying concentrations at 0°C for 18 hours.

$\square-\square$	D22, $I_{50} = 0.9$
$\bullet-\bullet$	D22 A19, $I_{50} = 4.5$
$\triangle-\triangle$	D22 61, $I_{50} = 4.5$

Fig 4.5

Inhibition of ATPase by venturicidin : O.L.III.

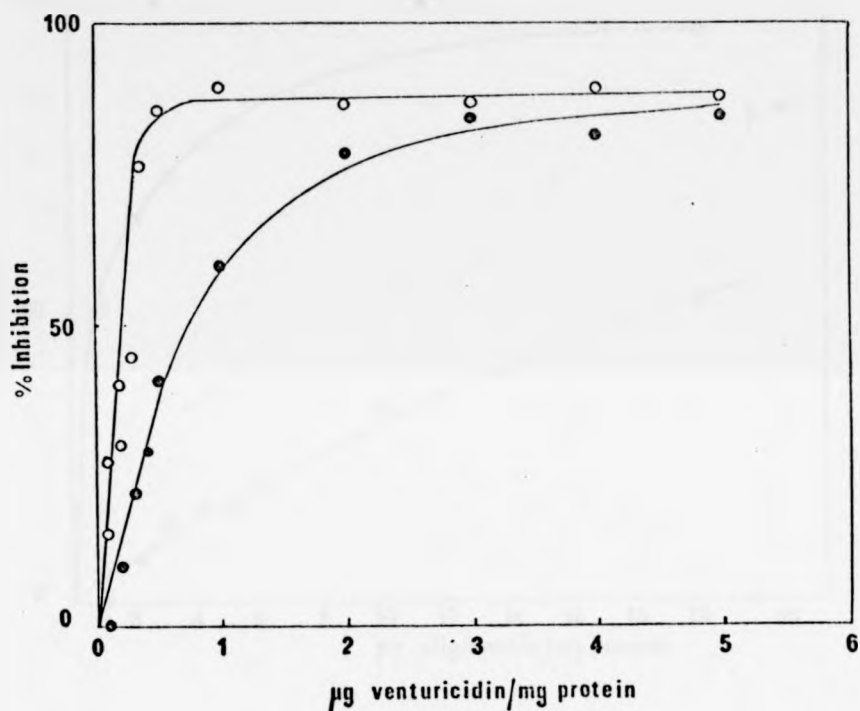


FIGURE 4.5.

ATPase assays were performed by the method of Wakabayashi and Gunge (163). Submitochondrial particles were preincubated with venturicidin for 5 minutes at 30°C.

○—○

D22, $I_{50} = 0.2$

●—●

D22 A19 $I_{50} = 0.75$

Fig 4.6

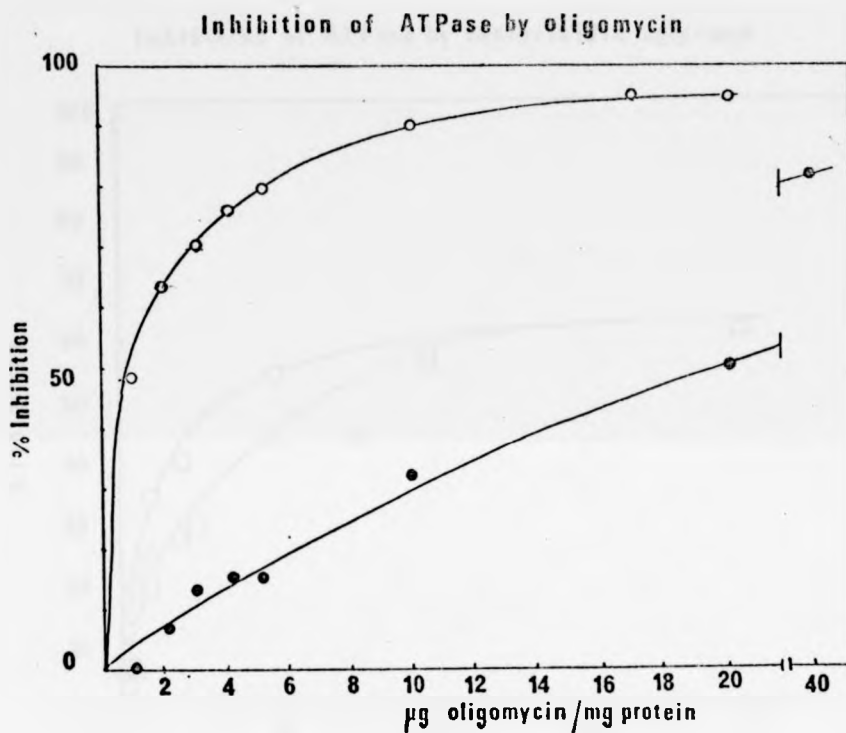


FIGURE 4.6.

ATPase assays were performed by the method of Wakabayashi and Gunge (163). Submitochondrial particles were preincubated with oligomycin for 5 minutes at 30°C.

○—○ D22, $I_{50} = 1.2$
 ●—● D22 A19 $I_{50} = 20$

Fig 4.7

Inhibition of ATPase by venturicidin aglycone

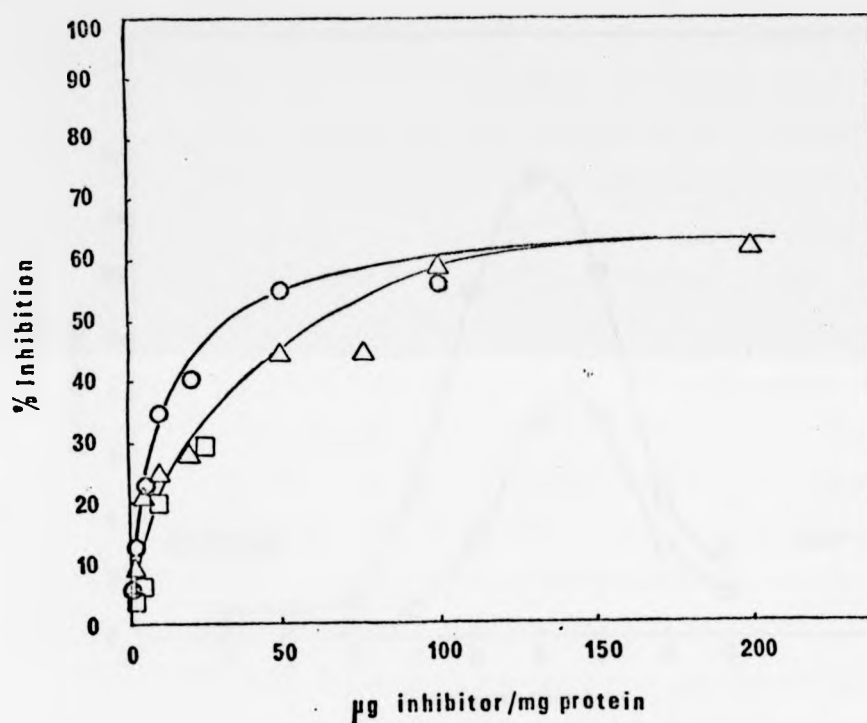


FIGURE 4.7.

ATPase assays were performed as described by Wakabayashi and Gunge (163). Submitochondrial particles were preincubated with venturicidin aglycone for 5 minutes at 30°C.

○—○	D22,	$I_{50} = 8$
△—△	D22 A21,	$I_{50} = 20$
□—□	D22 A19,	$I_{50} = 20$

Fig 4.8
Preparation of OS-ATPase: Density gradient centrifugation

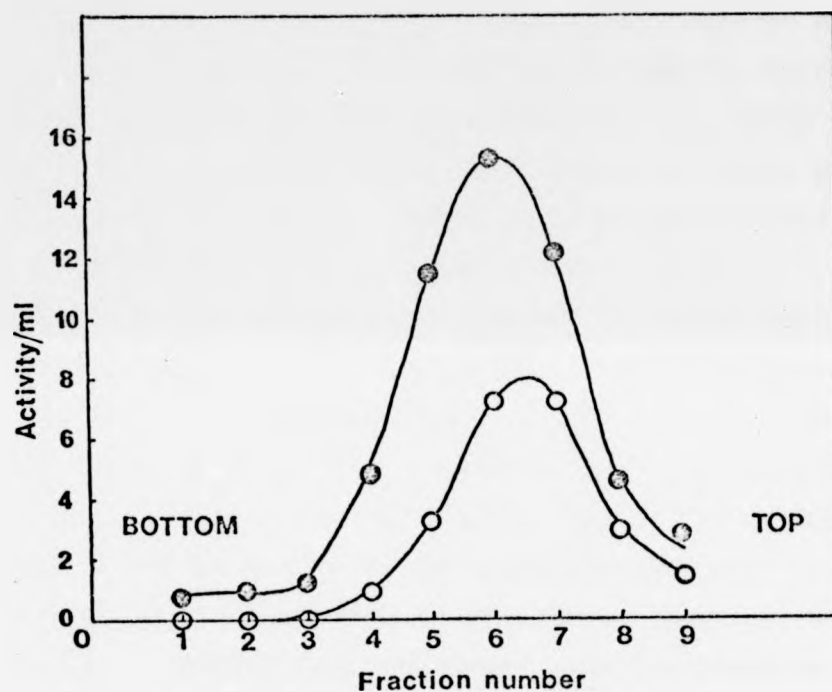


FIGURE 4.8.

Density gradient centrifugation of the triton extract of submitochondrial particles was performed as described by Tzagoloff (40) on a 5 - 20% sucrose gradient.

- ATPase activity in the absence of exogenous lipid.
- ATPase activity in the presence of Asolectin.

from D22 was titrated with a variety of lipids to determine the degree and specificity of any such stimulation. The results (Figs. 4.9 and 4.10, Table 4.2) indicate that the degree of stimulation by the various lipids is very similar, but the concentration of each lipid required for half maximal stimulation of the ATPase is markedly different. Ergosterol does not stimulate the ATPase at all, while maximal stimulation is given by the unsaturated fatty acids and by phosphatidic acid. Lowest stimulation is found with saturated fatty acids. There appears to be no structure-function relationship between the stimulatory and physical properties of the lipids. The stimulatory effect is due to a lowering of the K_m (ATP) of the enzyme as illustrated in Fig. 4.11. Although the lipids change the catalytic activity of the enzyme, they do not affect the inhibitor sensitivity at low concentrations, nor do they alter the pH dependence of the ATPase (Table 4.3 and Fig. 4.12). Accordingly, all assays were performed in the presence of a mixture of lipids at the concentration giving maximal stimulation as determined by titration as shown in Fig. 4.13. Reported ergosterol differences in the OS-ATPase of mutants and parental strains (183, 189) prompted an assay of this lipid in the mutants under investigation. As can be seen in Table 4.4, there is no correlation between the ergosterol content of the ATPase and the sensitivity to inhibitors of oxidative phosphorylation.

Titration of the OS-ATPase with DCCD shows that the resistance of the mutants is retained in the purified

Fig 4.9

Double reciprocal plot of titrations of OS-ATPase
with different fatty acids

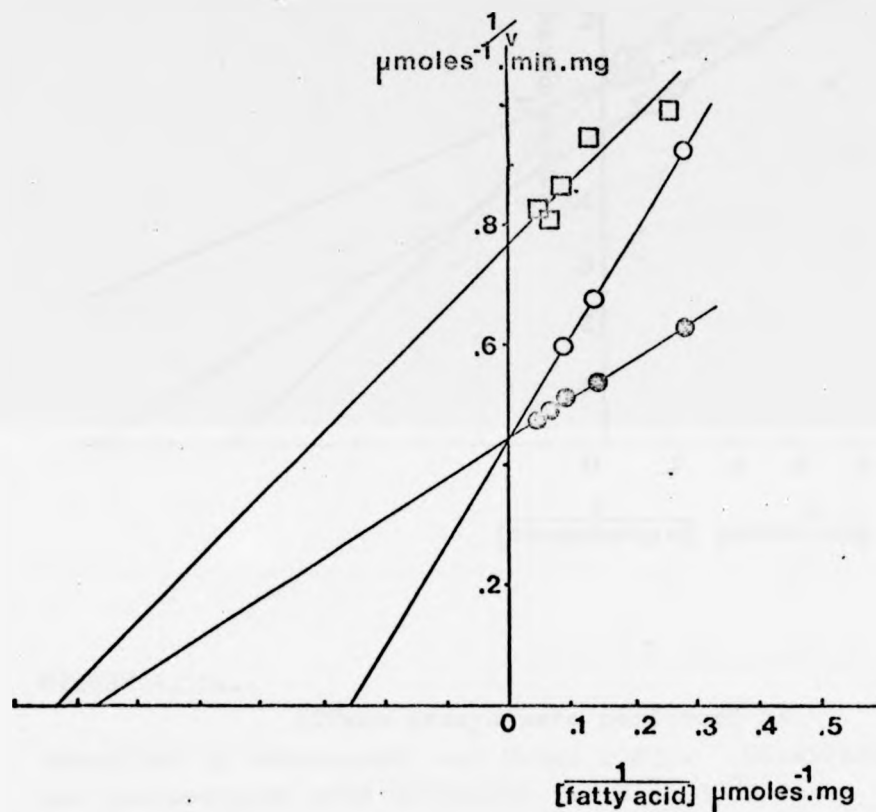


FIGURE 4.9.

ATPase assays were performed as described by Wakabayashi and Gunge (163). OS-ATPase was preincubated with sonicated lipid at 30°C for 5 minutes.

□—□ palmitic acid, ○—○ linoleic acid,
●—● oleic acid.

Fig 4.10a

Double reciprocal plot of titrations of OS-ATPase with different phospholipids

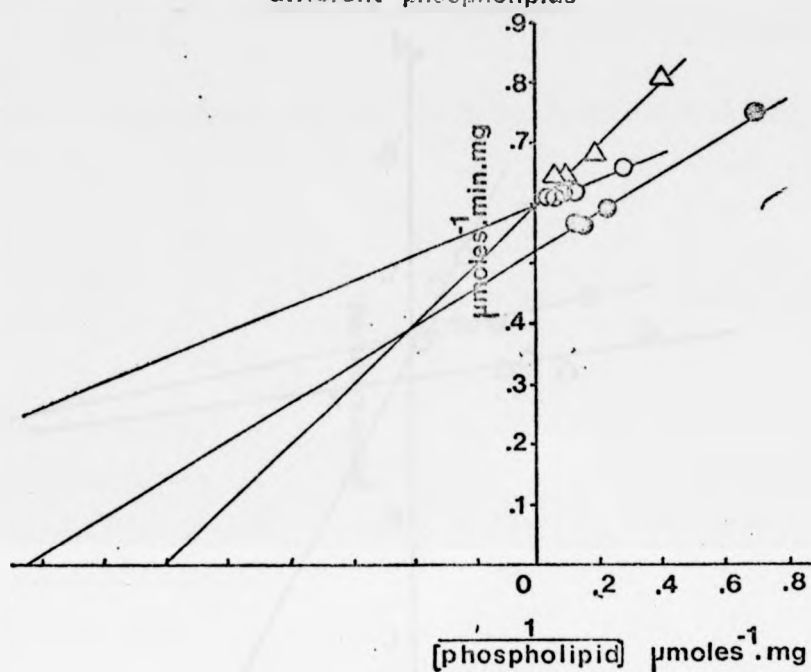


FIGURE 4.10a.

ATPase assays were performed as described by Wakabayashi and Gunge (163). OS-ATPase was preincubated with sonicated lipid at 30°C for 5 minutes.

- phosphatidylserine,
- lysophosphatidylcholine,
- △—△ phosphatidylcholine.

Fig 4.10b

Double reciprocal plot of titrations of OS-ATPase with different phospholipids.

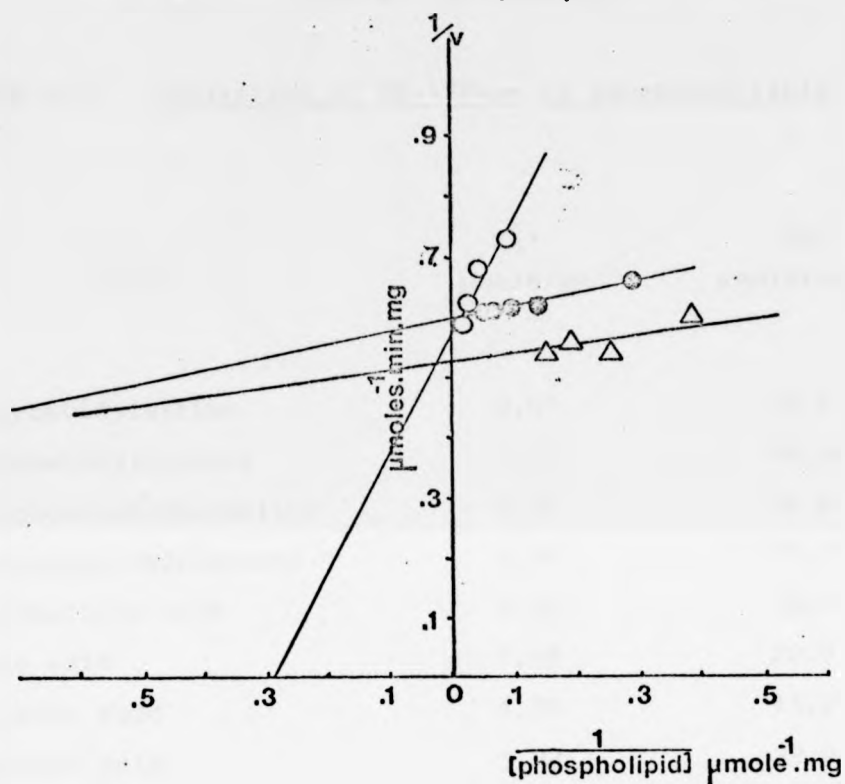


FIGURE 4.10b.

ATPase assays were performed as described by Wakabayashi and Gunge (163). OS-ATPase was preincubated with sonicated lipid at 30°C for 5 minutes.

- Δ—Δ phosphatidic acid,
- diphosphatidylglycerol,
- lysophosphatidylcholine.

TABLE 4.2. Activation of OS-ATPase by exogenous lipid

Lipid	K_a^* $\mu\text{mole/mg}$	V_m^* $\mu\text{mole/min/mg}$
phosphatidylserine	0.61	19.2
phosphatidylcholine	0.83	16.6
lysophosphatidylcholine	0.35	16.6
diphosphatidylglycerol	3.50	17.5
phosphatidic acid	0.31	19.0
oleic acid	1.48	22.7
palmitic acid	1.35	13.2
linoleic acid	3.92	22.7

* Data calculated from figures 4.9 and 4.10.

Fig 4.11

Lipid activation of ATPase

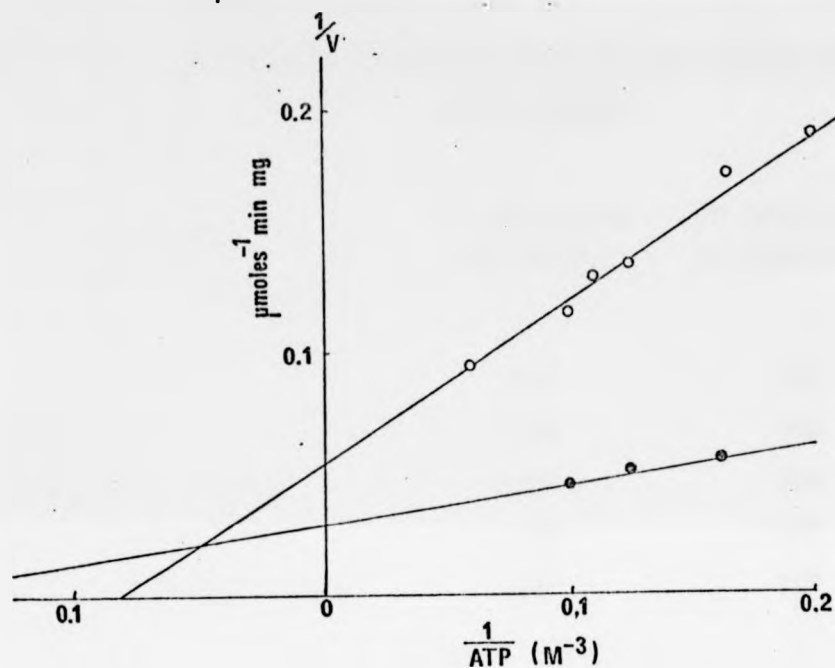


FIGURE 4.11.

ATPase assays were performed as described by Wakabayashi and Gunge (163). OS-ATPase was preincubated with oleic acid at 30°C for 5 minute.



OS-ATPase K_m (ATP) = 11.76mM



OS-ATPase + oleic acid K_m (ATP) = 3.6mM.

TABLE 4.3. Effect of exogenous lipid on the inhibition
of OS-ATPase

Lipid	% Inhibition by DCCD	% Inhibition by oligomycin
-	66%	52%
oleic acid	62%	50%
phosphatidic acid	65%	48%
phosphatidylserine	59%	50%
lysophosphatidylcholine	59%	45%

ATPase assays were performed as described by Wakabayashi and Gunge (163). OS-ATPase was preincubated with DCCD for 18 hours at 0°C, with oligomycin for 5 minutes at 30°C, and with lipids for 5 minutes at 30°C. Oligomycin concentration was 10µg/mg, DCCD concentration was 5µg/mg and lipids were added at their half maximal activation concentrations (see Table 4.2).

Fig 4.12

Effect of oleic acid on pH dependence of OS-ATPase

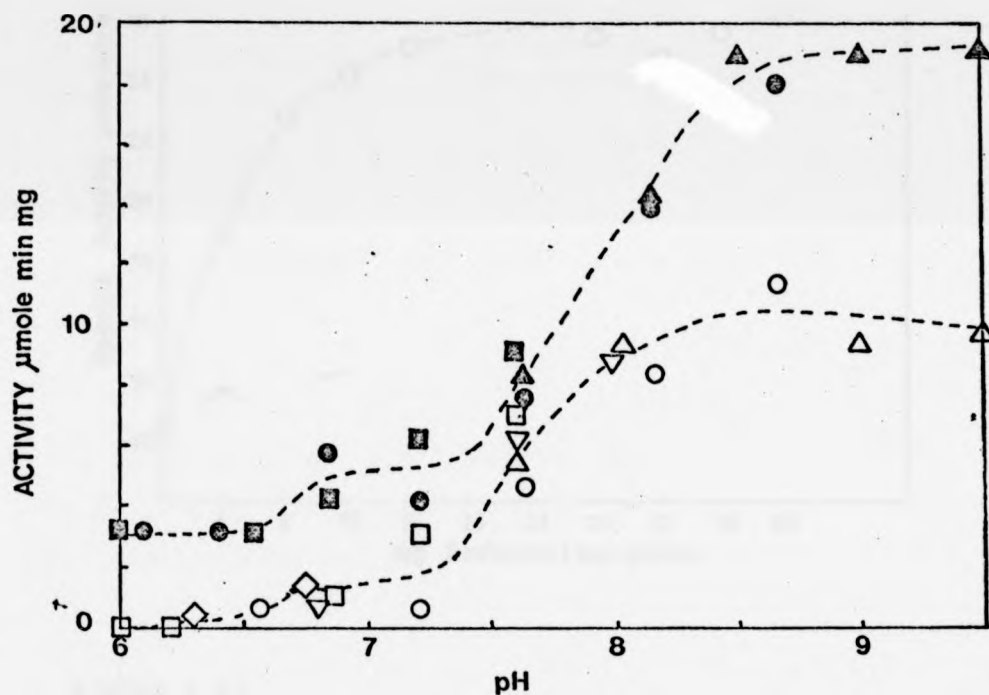


FIGURE 4.12.

ATPase assays were performed as described by Wakabayashi and Gunge (163). Open circles, ATPase without exogenous lipid; filled circles, ATPase activated by oleic acid,

□—□ Tris-maleate, ○—○ Tris-acetate,
 △—△ Tris-chloride, ◇—◇ Mes, ▽—▽ Hepes.

Fig 4.13

Activation of OS ATPase by Asolectin

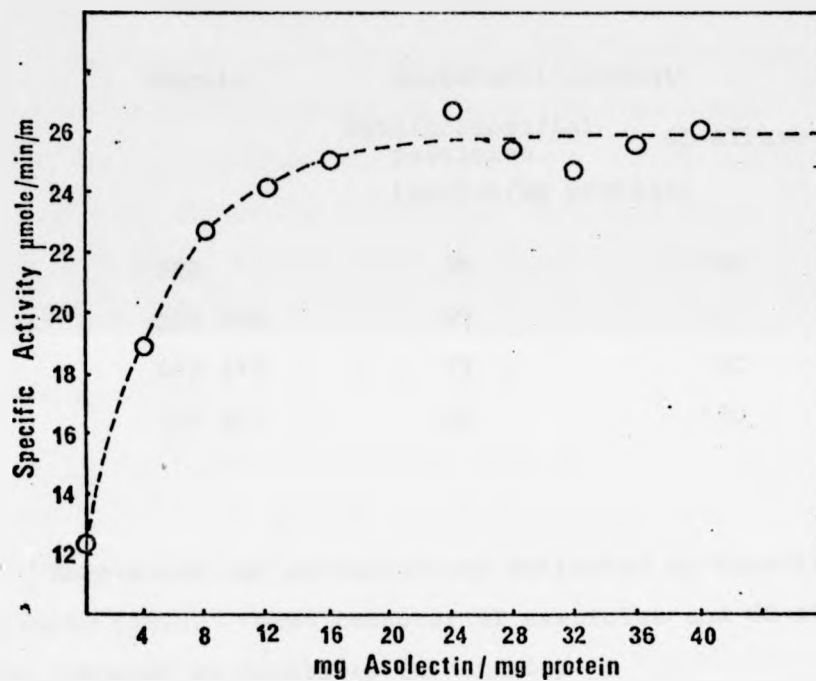


FIGURE 4.13.

ATPase assays were performed as described by Wakabayashi and Gunge (163). OS-ATPase was preincubated with asolectin at 30°C for 5 minutes.

TABLE 4.4. Ergosterol content of submitochondrial particles
and OS-ATPase

Strain	Ergosterol content	
	Submitochondrial particles (nmoles/mg protein)	OS-ATPase
D22	58	100
D22 A16	49	-
D22 A15	19	50
D22 A21	80	130

Ergosterol was extracted and estimated as described by Woods (187). Submitochondrial particles and OS-ATPase were prepared as described in Methods.

enzyme (Fig. 4.14). This is also the case for oligomycin and venturicidin. Moreover, the degree of resistance remains substantially the same whether the enzyme is membrane bound or not. The OL111 mutants D22, A19 and D2261 show a marked resistance to DCCD at the whole cell, mitochondrial and purified enzyme levels and were selected for further investigation, as these are also cross resistant to venturicidin. The mutant D22A21 was also selected for structural studies as this oligomycin resistant mutant is not cross resistant to DCCD or venturicidin.

4.4. Discussion

The investigative approach adopted in this laboratory is the isolation of mutants of S. cerevisiae resistant to inhibitors of oxidative phosphorylation and the subsequent selection of those mutants with an altered oligomycin sensitive ATPase. That this approach is applicable to the inhibitor DCCD is evident from the data provided by experiments conducted both at whole cell and subcellular levels. The value of such an inhibitor, with a well known chemistry (140), and reacting covalently with some specific membrane component (78), is that a subunit of the ATP synthetase altered in the selected mutants, may be specifically labelled and isolated. Moreover, as the step at which DCCD acts in the process of ADP phosphorylation is known, such a labelled component is implicated in the reactions at that step in the phosphorylation sequence. Oligomycin and DCCD have been presumed to act at the same

Fig 4,15

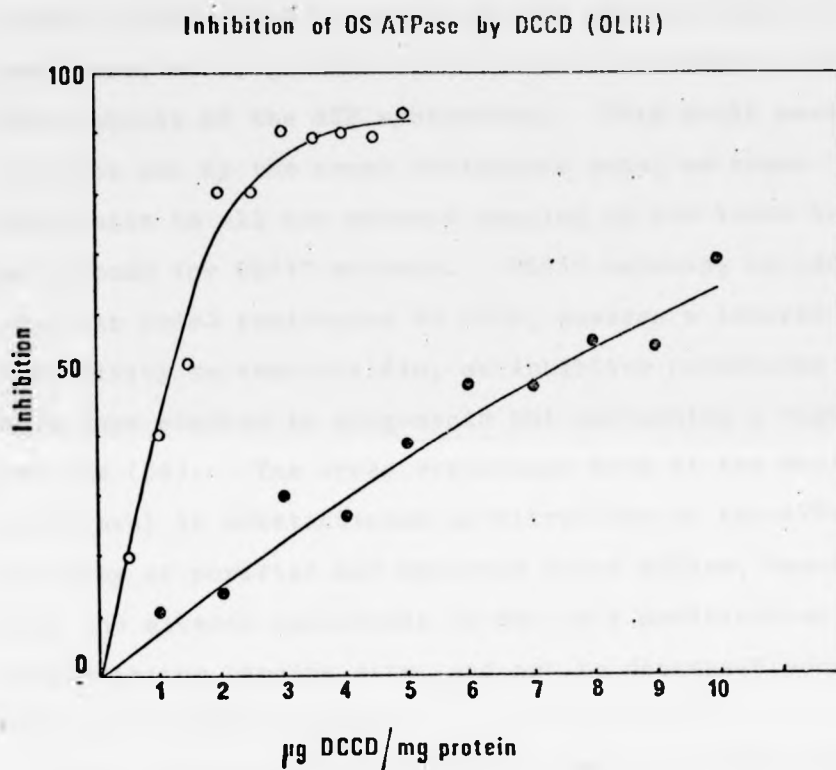


FIGURE 4.15.

ATPase assays were performed as described by Wakabayashi and Gunge (163). OS-ATPase was preincubated with varying amounts of DCCD at 0°C for 18 hours.

●—○ D22A19

○—○ D22

site (138, 139), in both inhibitory and stimulatory properties. It is unlikely that the inhibitors share a common binding site, considering the dissimilarity of structure, although both sites could be present on the same subunit of the ATP synthetase. This would seem to be borne out by the cross resistance data, as cross resistance to all the mutants mapping at one locus is only found for OL111 mutants. OL111 mutants, in addition to their cross resistance to DCCD, possess a lowered sensitivity to venturicidin, an inhibitor possessing a structure similar to oligomycin but containing a sugar residue (34). The cross resistance data at the whole cell level is substantiated by titrations of the ATPase activity of purified and membrane bound ATPase, demonstrating that the altered resistance is due to a modification of the inhibitor binding site, and not to decreased permeability or detoxification.

The inhibition curves for the membrane bound ATPase also provide some information on the binding of venturicidin. The OL111 mutant A19 is resistant to oligomycin, venturicidin and DCCD. The OL1 mutant D22A21 is resistant to oligomycin but has no cross resistance to venturicidin and DCCD. The inhibitor Venturicidin X, the aglycone of venturicidin, acts like oligomycin rather than venturicidin in that the mutants D22A19 and D22A21 are both resistant to the aglycone, and also in the high concentrations of inhibitor required for maximal inhibition. This implies that there are two binding sites for venturicidin, one similar (or identical) to the oligomycin binding site and

one in which the rhamnose sugar is involved in binding.

The Triton X-100 solubilised OS-ATPase described by Tzagoloff (40) has the advantages of being an easily purified, homogeneous preparation, in contrast to the other OS-ATPase complexes described in the literature (58 - 62). In addition, the preparation of such a complex from a facultative anaerobe such as S. cerevisiae, which may be genetically or phenotypically manipulated to produce controlled modifications of the complex, provides the mitochondriologist with a powerful research tool for the investigation of the mechanism of ATP synthesis. It is somewhat surprising therefore, that the properties of the OS-ATPase, in particular the activation by lipids and the effect of such stimulation on the inhibitor sensitivity of the enzyme, have not been described in the literature, although corresponding work on the beef heart ATPase has been reported (40, 58 - 64). In agreement with the findings of Swanljung (64) and Cunningham (63) on the beef heart OS-ATPase, all phospholipids and fatty acids tested are competent to stimulate ATPase activity. However, the yeast OS-ATPase differs from the beef heart enzyme in that maximal activation is provided by phosphatidic acid and not by lysolecithin, which is the poorest activator of all of the phospholipids, giving the lowest maximal activation.

Swanljung (63) has shown that addition of phospholipid to the lipid depleted OS-ATPase from beef heart mitochondria lowers the K_m (ATP). Cunningham (64) however, has shown that although exogenous phospholipid

increases V_{\max} of CF_0F_1 , the K_m (ATP) is identical in unactivated and asolectin treated preparations. The yeast OS-ATPase is similar to that of Swanlung in that V_{\max} is increased and K_m (ATP) is lowered on addition of lipid. The mechanism by which the lipid activates the ATPase complex is suggested by the finding by Cunningham that diglycerides do not stimulate CF_0F_1 (64). Verkleij (65) has shown that diglycerides do not form a bilayer structure in aqueous solution, unlike phospholipids and fatty acids. It is possible therefore that the OS-ATPase must be incorporated into a lipid bilayer for maximal activity, in accordance with the chemiosmotic hypothesis of Mitchell(3) of which the major premise is the orientation of the ATPase in the membrane. Ergosterol, one of the major components of the OS-ATPase, does not stimulate the OS-ATPase activity, nor does it readily form vesicles. It is perhaps surprising that although phospholipids and fatty acids affect the kinetic properties of the OS-ATPase, exogenous phospholipid has no effect on either pH dependance or inhibitor sensitivity, especially in view of the data presented in Chapter 3. However, the OS-ATPase as prepared contains 10% endogenous lipid (40), and it may be that this strongly bound phospholipid plays an important role in the functioning of the ATPase. Moreover, the loss in inhibitor sensitivity found in Chapter 3 is due to a drastic change in the fluidity of the membrane, causing a change in the conformation of the OS-ATPase: the endogenous lipid may fulfil the function of holding the enzyme in the conformation which is inhibitor sensitive.

CHAPTER 5

Investigation of the DCCD binding site in *S. cerevisiae*

5.1. Introduction

DCCD has been shown to inhibit energy linked reactions in mitochondrial preparations derived from both rat liver and beef heart (32), and to bind covalently and specifically with a low molecular weight proteolipid at comparable levels to those producing inhibition. Avner and Peers (190) found that ^{14}C DCCD only binds to yeast mitochondria at levels much lower than those required for inhibition. Broughall (152) has confirmed and extended these observations and has found that most of the applied inhibitor is only recoverable as the hydration product dicyclohexylurea (DCU). By analogy with the proposed mechanism of DCCD inhibition in red blood cell ATPase (191), Broughall has suggested that DCCD does bind covalently to yeast mitochondrial membranes, but that the resulting "activated carboxyl" is unstable due to the proximity of a membrane nucleophile, which forms a peptide bond and releases DCU (152). Some evidence, using the external nucleophile glycine ethyl ester, has been produced to support this hypothesis.

It has long been known that the composition of the mitochondrial membrane is extensively modified by environmental factors such as availability of oxygen or catabolite repression (188, 192). It is also possible to deplete the mitochondrial membrane of specific proteins by treatment with chaotropic agents such as urea and

detergents. It should be possible therefore to modify the yeast mitochondrial membrane in such a way as to decrease the cleavage of the DCCD-membrane adduct and to identify the species responsible for inhibitor binding. Such an approach, if combined with manipulation of the mitochondrial protein synthesis system with appropriate inhibitors such as cycloheximide and chloramphenicol should also give valuable information on the biogenesis of the ATPase complex.

5.2. Methods and Materials

Yeast growth and preparation of mitochondria

Aerobic and anaerobic growth of S. cerevisiae strain D22 (ad₂,a) and the preparation of mitochondrial membranes were as previously described (186). Mitochondria and promitochondria were further purified by sucrose density centrifugation. Y_{ETP} (NaBr) and Y_{ETP} (NaBr, NH₄OH) were prepared as described by Tzagoloff (70).

Binding of ¹⁴C DCCD

Submitochondrial particles (2mg) were incubated in 5ml 250mM Sucrose, 10mM Tris-acetate pH 7.5 at 0°C with varying amounts of DCCD. After 18 hours incubation, the suspension was centrifuged at 100,000g for 30 minutes and the pellet counted by liquid scintillation after dissolution in 1ml 2% (w/v) Triton X-100.

Assay procedures

ATPase activity was assayed by the method of Wakabayashi and Gunge (163), phosphate was estimated by

the method of Fiske and SubbaRow (164), modified as described in 4.2. Protein was estimated by the biuret method of Gornall et al. (141), in the presence of 1% deoxycholate.

DCCD/DCU mixtures were separated on Alumina plates (Merck, aluminium sheets) in Benzene/Ethyl acetate (9:1 v/v). Chloroform/Methanol (2:1 v/v) extraction of mitochondrial membranes was carried out as described by Cattell et al. (78). Triton extraction and precipitation with antisera to OS-ATPase was performed as described by Tzagoloff (40). Antisera to OS-ATPase was obtained from rabbits. Rabbits were immunised with 8mg OS-ATPase according to Schatz et al. (35).

Materials

^{14}C DCCD was a kind gift from Prof. R.B. Beechey (Shell Research Ltd., Sittingbourne). DCCD was purchased from BDH. ^3H leucine was purchased from the Radiochemical Centre (Amersham). 4-Amino-2,2,6,6-tetramethylpiperid-1-yloxy was purchased from R. Emanuel Ltd. All other reagents were analytical grade.

5.3. Results

Binding of ^{14}C DCCD to aerobic mitochondria

Incubation of ^{14}C DCCD with mitochondria, submitochondrial particles and isolated OS-ATPase from S. cerevisiae grown aerobically on ethanol gave negligible binding of the inhibitor as shown in Table 5.1.

TABLE 5.1. Binding of ^{14}C DCCD to mitochondrial membranes
from aerobically grown yeast

	nmoles/mg DCCD	% bound
Mitochondria	1.0	3.1
SMP	1.0	3.3
OS-ATPase	3.0	5.0

^{14}C DCCD was incubated with the membrane preparations at the concentrations shown at 0°C for 18 hours. The mitochondria and submitochondrial particles were centrifuged down, the OS-ATPase was precipitated with an equal volume of 10% TCA. Radioactivity was counted by liquid scintillation in Triton/toluene/butyl PBD.

Loss of DCCD sensitivity

As can be seen in Fig. 5.1., the inhibitory potency of DCCD is diminished by preincubation of the submitochondrial particles with a nucleophilic spin probe (4-amino-2,2,6,6-tetramethylpiperid-1-yloxy). The degree of inhibition is dependant on the concentration of nucleophile at any given concentration of DCCD (Fig. 5.2.). This reduction in potency is not due to direct reaction of nucleophile with DCCD as shown by TLC of DCCD, spin probe, and a combination of the two on alumina plates in Benzene/Ethyl Acetate (9:1 v/v). Although this displacement of DCCD from the membrane was expected to be accompanied by a concomitant binding of nitroxide label, no E.S.R. signal could be detected. Reduction of the probe cannot be ruled out, even though incorporation was carried out in the presence of ferricyanide. Alternatively, the probe may be almost totally immobilised (as in the case of NCCD (179)), and the signal too broadened to detect at the low levels of probe used.

Binding of DCCD to Y_{ETP} (NaBr, NH_4OH)

Depletion of F_1 from yeast submitochondrial particles increases the degree of binding of ^{14}C DCCD from 3% to 38%, and depletion of both F_1 by NaBr treatment, and OSCP by NH_4OH treatment increases the degree of binding to almost 50% as shown in Table 5.2. As can be seen in Fig. 5.3., this increased binding of DCCD to Y_{ETP} (NaBr, NH_4OH) is due in part to the existence of a high affinity site which is not detectable in normal aerobic mitochondrial

Fig 5.1

Inhibition of ATPase in YETP by DCCD

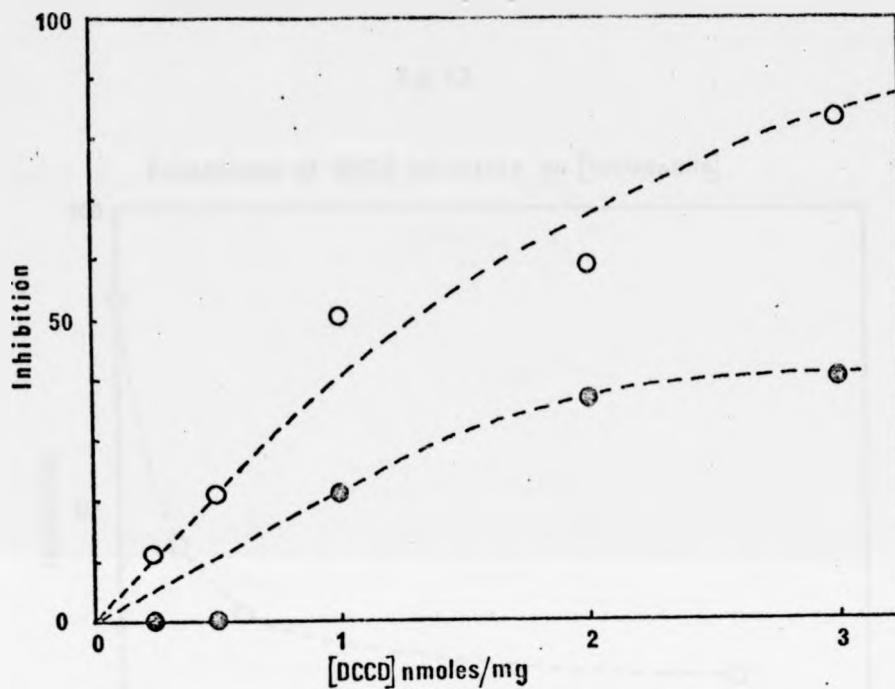


FIGURE 5.1.

ATPase assays were performed as described by Wakabayashi and Gunge (163). DCCD and 4-amino-2,2,6,6 tetramethylpiperid-1-yloxy were preincubated with yeast submitochondrial particles for 18 hours at 0°C.

- No spin probe
 ●---● 1nmole spin probe/mg protein.

Fig 5.2

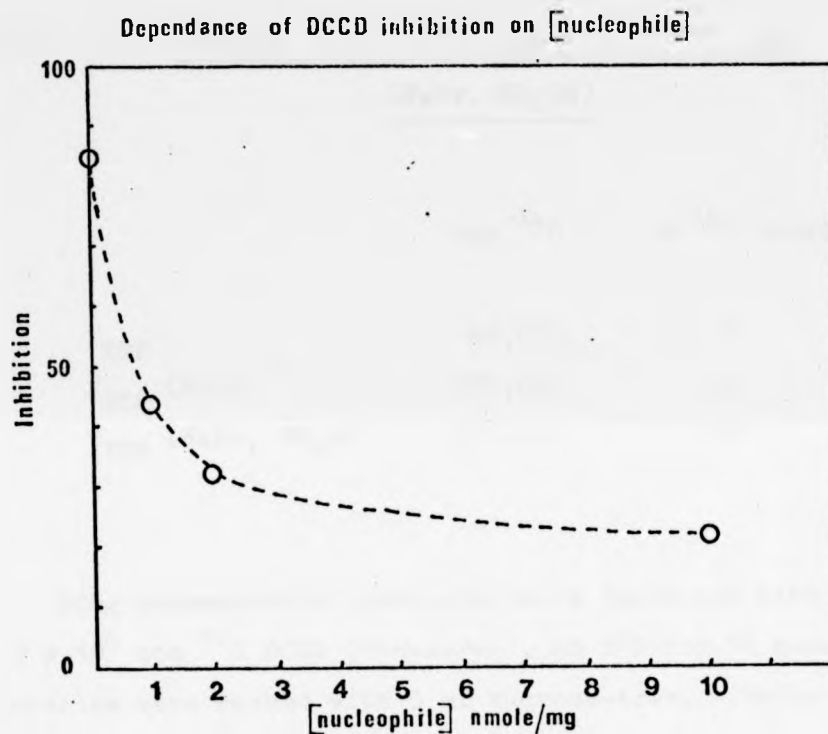


FIGURE 5.2.

ATPase assays were performed as described by Wakabayashi and Gunge (163). DCCD and 4-amino 2,2,6,6 tetramethylpiperid-1-yloxy were preincubated with yeast submitochondrial particles for 18 hours at 0°C. DCCD concentration was 2nmoles/mg protein.

TABLE 5.2. Binding of DCCD to Y_{ETP} (NaBr) and Y_{ETP}
(NaBr, NH_4OH)

	cpm ^{14}C	% ^{14}C bound
Y_{ETP}	60,000	3
Y_{ETP} (NaBr)	835,000	38
Y_{ETP} (NaBr, NH_4OH)	917,000	49

20mg mitochondrial particles were incubated with 2×10^6 cpm ^{14}C DCCD (1nmole/mg), at $0^\circ C$ for 18 hours. Particles were washed with 5 ml sucrose-tris. Radioactivity was determined by liquid scintillation in Triton/toluene/butyl PBD.

Fig 5.3

BINDING OF DCCD TO YETP(NaBr, NH₄OH): OL^r AND OL^s.

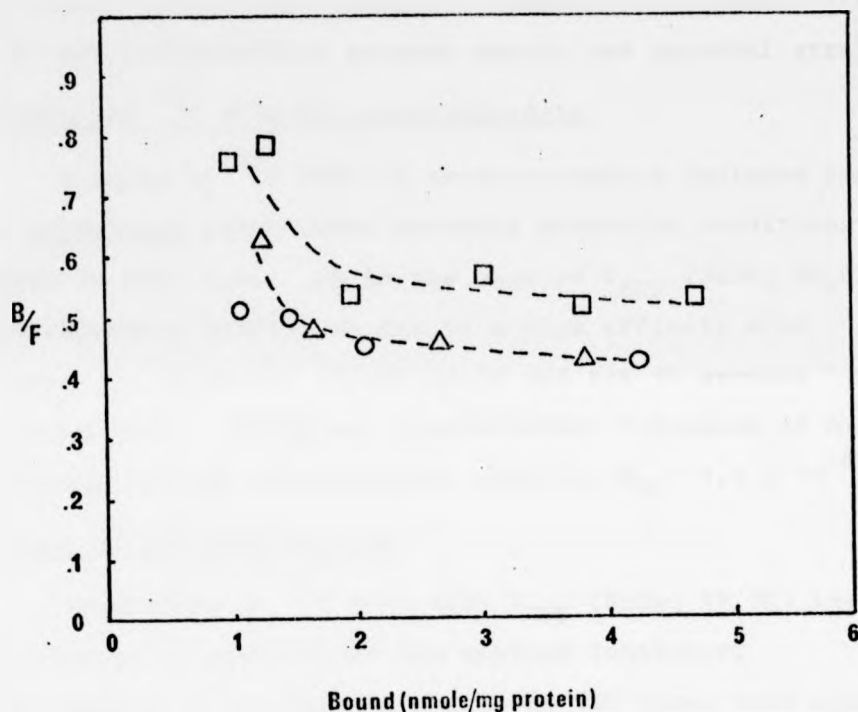


FIGURE 5.3.

Y_{ETP} (NaBr, NH₄OH) were prepared as described by Tzagoloff (70). Depleted particles (2mg) were incubated with varying amounts of DCCD at 0°C for 18 hours in 5ml 250mM Sucrose, 10mM Tris-acetate pH 7.5. The suspension was centrifuged at 100,000g for 30 minutes and the pellet counted by liquid scintillation after solubilisation in 2% triton X-100.

△—△ D22, □—□ D22 A19, ○—○ D22 A21.

membranes. No difference could be detected in the low affinity site between D22 parental and the DCCD resistant mutant D22 A19, and the specific activity of the ^{14}C DCCD available is not high enough to resolve the high affinity site and differentiate between mutant and parental strain.

Binding of ^{14}C DCCD to promitochondria

Binding of ^{14}C DCCD to promitochondria isolated from S. cerevisiae grown under strictly anaerobic conditions is shown in Fig. 5.4. As in the case of Y_{ETP} (NaBr , NH_4OH), the increased binding is due to a high affinity site present in anaerobic mitochondria and not in aerobic mitochondria. These are approximately 1.5nmoles of high affinity site/mg mitochondrial protein, $K_D = 1.7 \times 10^{-6}\text{M}$.

Nature of the binding site

Incubation of ^{14}C DCCD with Y_{ETP} (NaBr , NH_4OH) leads to binding of some 50% of the applied inhibitor. Examination of the unbound counts by TLC shows that approximately half of the unbound inhibitor is present in the supernatant as DCU, and half as protein which does not migrate: no counts are found in the position corresponding to DCCD (Fig. 5.5.). Chloroform/methanol extraction of the washed DCCD treated Y_{ETP} (NaBr , NH_4OH) removes 95% of the bound counts, and TLC of this extract is shown in Fig. 5.6. The inhibitor is predominantly present as a species which does not migrate in Benzene/Ethyl acetate (9:1 v/v) with only small amounts of DCCD and DCU. Lipids migrate easily in this solvent system, while proteins do not.

Fig 5.4

BINDING OF DCCD TO YETP FROM AEROBIC AND ANAEROBIC YEAST

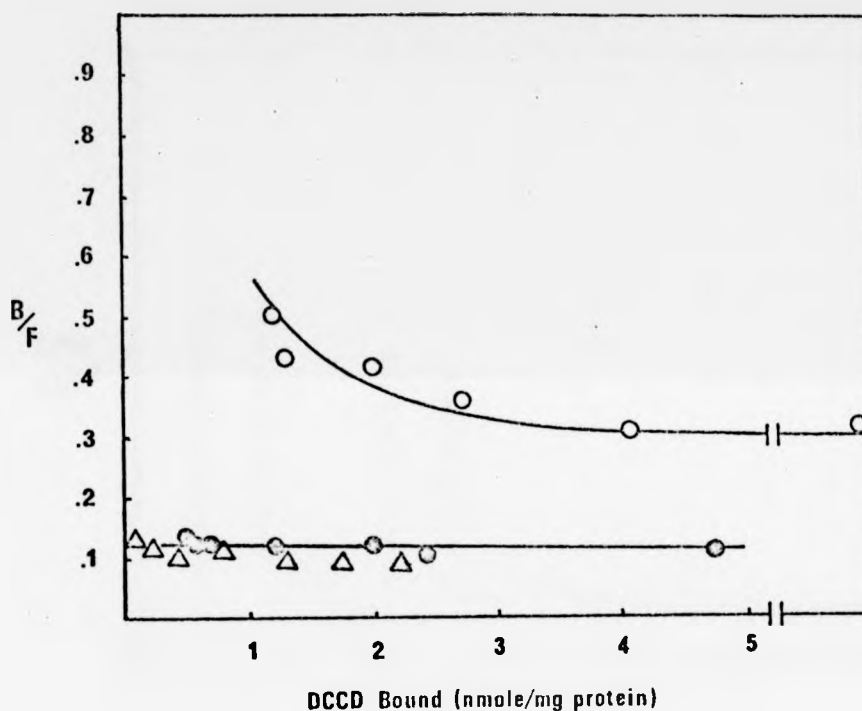


FIGURE 5.4.

Promitochondria and mitochondria were prepared as in Methods. Depleted particles (2mg) were incubated with varying amounts of DCCD at 0°C for 18 hours in 5ml 250mM Sucrose, 10mM Tris-acetate pH 7.5. The suspension was centrifuged at 100,000g for 30 minutes and the pellet counted by liquid scintillation after solubilisation in 2% triton X-100.

- promitochondria
- mitochondrial band (aerobic)
- △—△ mitochondrial band (anaerobic).

Fig 5.5

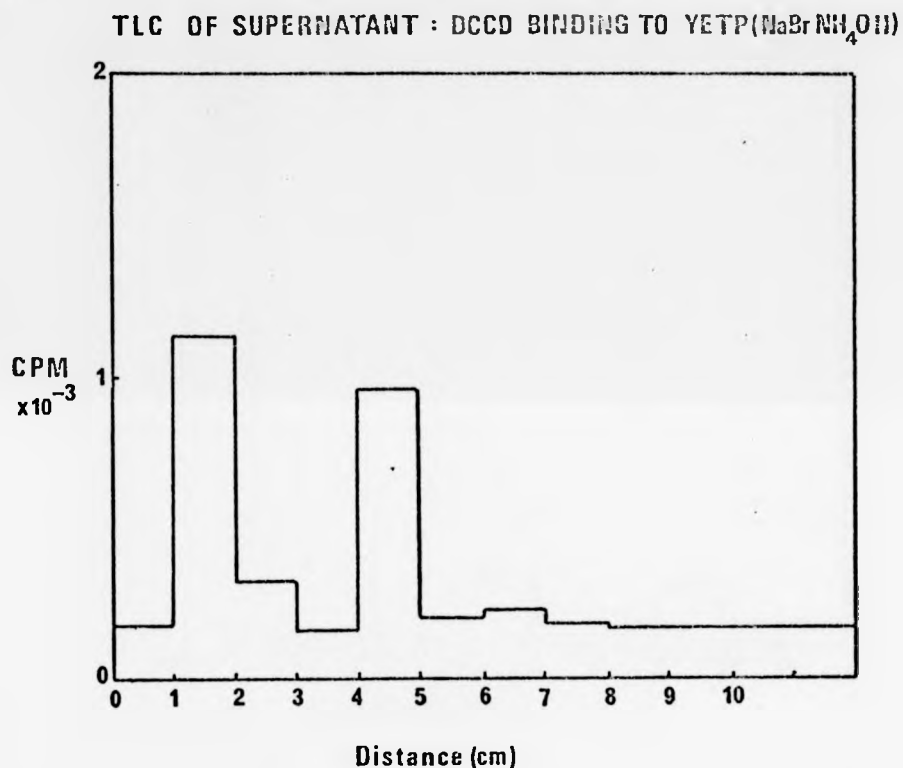


FIGURE 5.5.

¹⁴C DCCD was incubated with depleted yeast submitochondrial particles at 2nmole/mg, for 18 hours at 0°C. The suspension was centrifuged at 100,000g for 30 minutes and the supernatant extracted with chloroform-methanol (2:1 v/v). The extract was reduced in volume on a rotary evaporator and run on an aluminium oxide TLC plate in benzene/ethyl acetate (9:1 v/v).

Fig 5.6

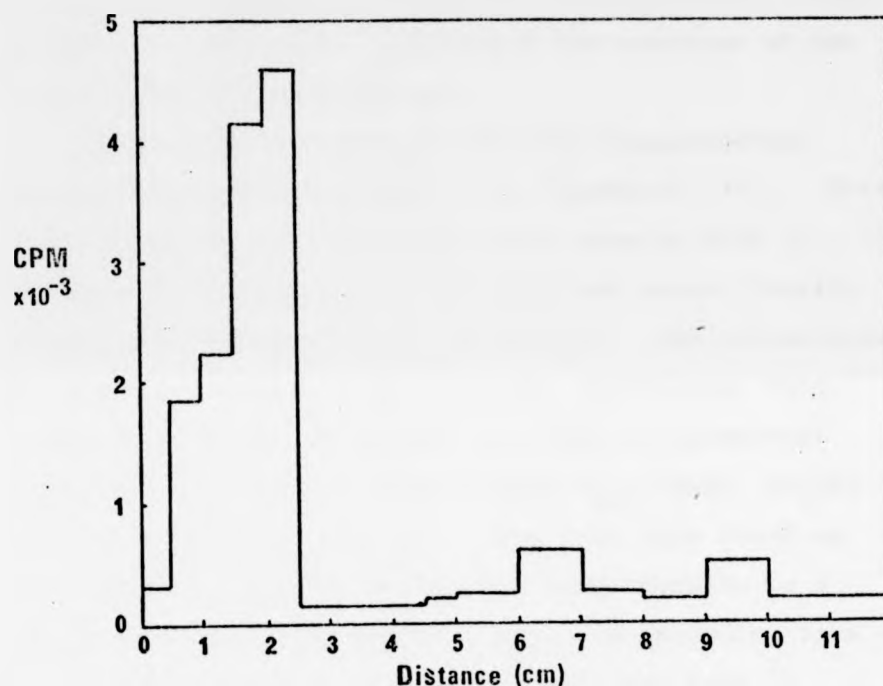
T.L.C : CHLOROFORM METHANOL EXTRACT OF YETP (NaBr, NH₄OH)

FIGURE 5.6.

¹⁴C DCCD was incubated with Y_{ETP} (NaBr, NH₄OH) for 18 hours at 0°C. The particles were centrifuged down at 100,000g and extracted for 18 hours with 20 volumes chloroform/methanol (2:1 v/v). The extract was reduced in volume on a rotary evaporator and run on an aluminium oxide TLC plate in benzene/ethyl acetate (9:1 v/v).

Triton X-100 extraction of both Y_{ETP} (NaBr, NH_4OH) and anaerobic mitochondrial membranes solubilises the membrane bound inhibitor, and this solubilised inhibitor may be precipitated by antisera to the OS-ATPase complex as shown in Table 5.3., indicating the presence of the binding site in the OS-ATPase.

3H leucine was incorporated into mitochondrial membrane proteins as described by Tzagoloff (70). Triton X-100 extraction of these membranes removed some 10 - 15% of the total radioactivity and this was almost totally precipitable by antisera to OS-ATPase. SDS-polyacrylamide gel electrophoresis of the antisera precipitate from triton extracts of 3H leucine labelled mitochondrial membranes and from ^{14}C DCCD treated Y_{ETP} (NaBr, NH_4OH) are shown in Figs. 5.7. and 5.8. The only band found on the latter gel runs at a position corresponding to a protein of 43,000 molecular weight, corresponding to a consistently observed peak in the antisera precipitate gel from 3H labelled mitochondria. Tzagoloff has identified this band as an incompletely solubilised polymeric form of the proteolipid subunit 9 (76). Conversion of this polymeric form to the monomer may be accomplished by treatment with alkali, which is not possible in this case, due to the lability of the DCCD-protein bond.

Biogenesis of the DCCD binding site

Inhibition of ATPase activity in mitochondria prepared from derepressing cells is almost invariant as shown in Fig. 5.9. S. cerevisiae D22 was grown in 5.4% glucose

TABLE 5.3. Precipitation of triton extracts with antisera to OS-ATPase

	¹⁴ C DCCD (cpm)		
	Triton extract	Antisera precipitate	% precipitated
Promitochondria	11,860	4,350	37
Y _{ETP} (NaBr, NH ₄ OH)	38,900	26,821	69

¹⁴C DCCD was incubated at 2nmole/mg with promitochondrial particles or Y_{ETP} (NaBr, NH₄OH) for 18 hours at 0°C. Washed particles were extracted with 0.5% Triton X-100 and antisera precipitates were prepared as described by Tzagoloff (40). Precipitates were dissolved in 2% triton X-100 and counted in triton/toluene/butyl PBD.

TABLE 5.3. Precipitation of triton extracts with antisera
to OS-ATPase

	¹⁴ C DCCD (cpm)		
	Triton extract	Antisera precipitate	% precipitated
Promitochondria	11,860	4,350	37
Y _{ETP} (NaBr, NH ₄ OH)	38,900	26,821	69

¹⁴C DCCD was incubated at 2nmole/mg with promitochondrial particles or Y_{ETP} (NaBr, NH₄OH) for 18 hours at 0°C. Washed particles were extracted with 0.5% Triton X-100 and antisera precipitates were prepared as described by Tzagoloff (40). Precipitates were dissolved in 2% triton X-100 and counted in triton/toluene/butyl PBD.

Fig 5.7

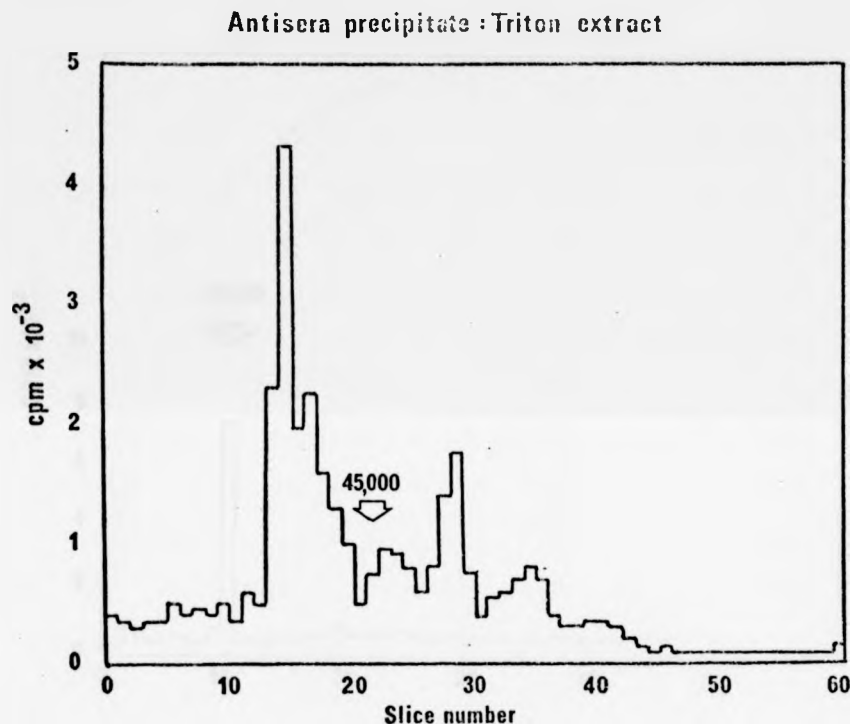


FIGURE 5.7.

D22 *S. cerevisiae* were grown for 12 hours in a 5.4% glucose medium. The cells were harvested and transferred to a medium containing 2mg/ml chloramphenicol and 0.8% glucose. After aeration for 6 hours, the harvested cells were grown in a medium containing 0.8% glucose, 10^{-5} M cycloheximide and 1.25 μ Ci 3 H leucine/ml. for 20 hours. The cells were harvested and washed. Mitochondria and submitochondrial particles were prepared as described in Methods. Triton extraction and precipitation with OS-ATPase antisera were performed as described by Tzagoloff (70). Polyacrylamide gel electrophoresis was as described by Weber and Osbourne (143).

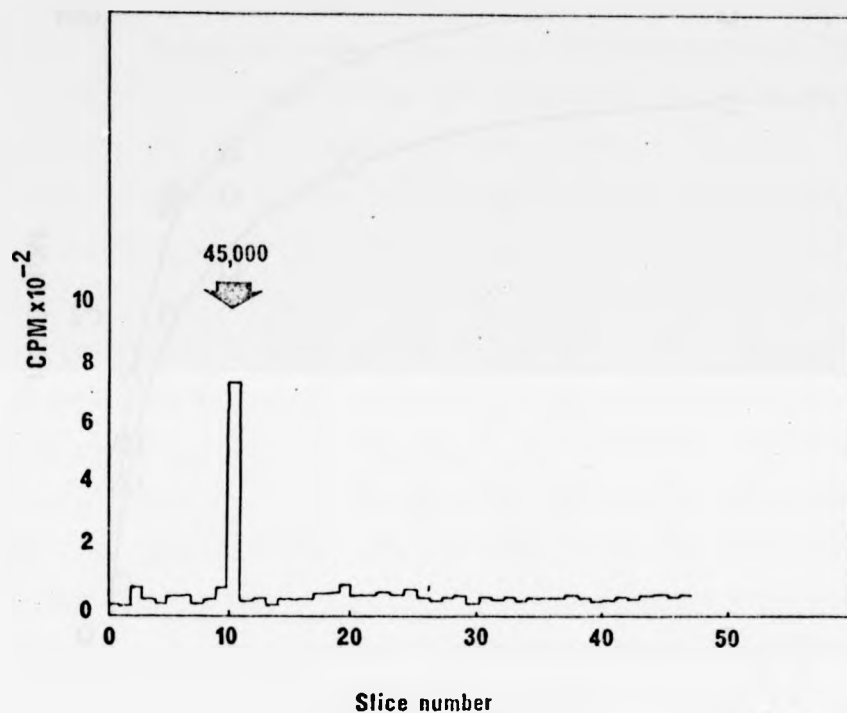


FIGURE 5.8.

SDS-gel electrophoresis of the DCCD binding site in Y_{ETP} (NaBr, NH_4OH)

Y_{ETP} (NaBr, NH_4OH) were prepared as described by Tzagoloff (70) and incubated at $0^\circ C$ for 18 hours with ^{14}C DCCD. Triton extraction and antisera precipitation were as described by Tzagoloff (40, 70). Polyacrylamide gel electrophoresis was as described by Weber and Osbourne (143).

Fig 5.9

Derepression of D22.

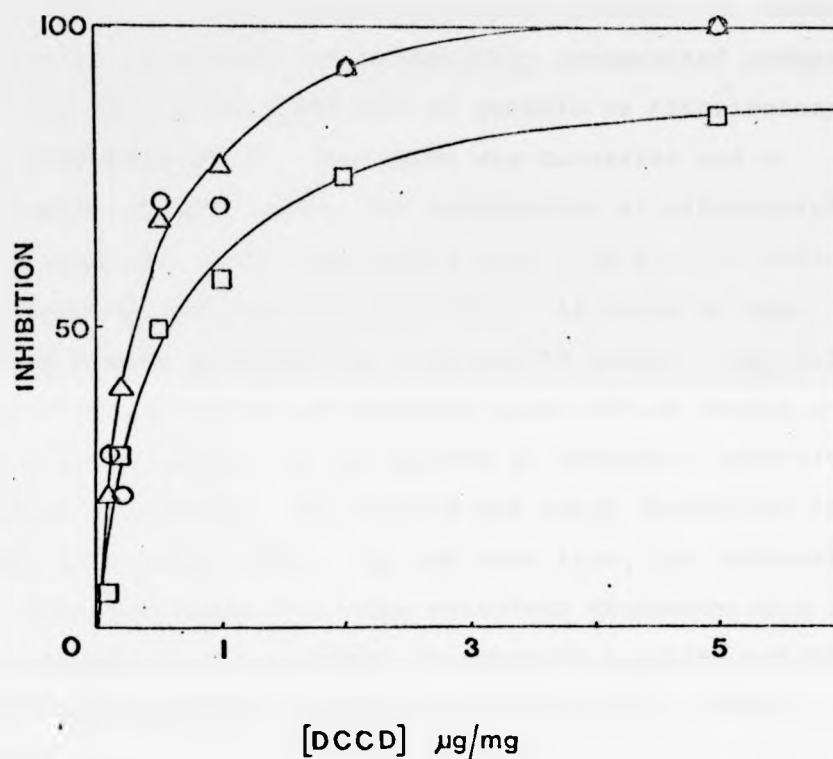


FIGURE 5.9.

Derepression of yeast in the presence of chloramphenicol and cycloheximide was carried out by the method outlined in the text. DCCD was preincubated with submitochondrial particles for 18 hours at 0°C. ATPase assays were performed as described by Wakabayashi and Gunge (163).

□—□ 0 hours, ○—○ 3 hours, △—△ 6 hours.

for twelve hours, and the glucose repressed cells were harvested and transferred to fresh culture medium containing chloramphenicol (2mg/ml) and 0.8% glucose and aerated for six hours. Under these conditions synthesis of essential (glucose repressed) cytoplasmically synthesised proteins is initiated, but synthesis of protein on mitoribosomes is inhibited (91). The yeast was harvested and a suitable aliquot broken for preparation of mitochondria, the remainder being inoculated into 0.8% glucose medium containing cycloheximide (10^{-5} M). Aliquots of the culture were harvested at 3, 6 and 18 hours. I_{50} values for DCCD inhibition of membrane bound ATPase change only by a small amount but the amount of inhibitor sensitive ATPase increases. At 0 hours the total inhibition is 80%, at 6 hours 100%. At the same time, the proportion of unincorporated F_1 in the cytoplasm decreases at a rate comparable to a concurrent increase in specific activity of DCCD sensitive membrane bound ATPase, as shown in Table 5.4.

Qualitatively the same results are obtained on derepression of the oligomycin mutants D22 A19 (Fig. 5.10.) and D22 A21 (Fig. 5.11.). In both cases, the I_{50} values for DCCD inhibition are approximately the same during derepression, slightly more inhibitor being required at 0 hours. The degree of inhibition is consistently lower at 0 hours, although this is more pronounced in the case of D22 A19. Resistance and sensitivity to DCCD is maintained throughout. Derepression of D22 in the absence of chloramphenicol or cycloheximide gives a similar result

TABLE 5.4. Biogenesis of OS-ATPase

Time after derepression (hours)	Aurovertin sensitive ATPase $\mu\text{mole min}^{-1} \text{mg}^{-1}$	
	submitochondrial particles	post-ribosomal supernatant
0	1.3	0.08
1	1.8	0.06
3	2.85	0.032

S. cerevisiae D22 was grown in 5.4% glucose medium for 12 hours. The cells were harvested and transferred to 0.8% glucose medium containing 2mg/ml chloramphenicol. After 6 hours growth, the cells were harvested, washed and transferred to 0.8% glucose medium containing 10^{-5}M cycloheximide. Aliquots were harvested for preparation of submitochondrial particles at 0, 1, 3 hours. Post-ribosomal supernatants were centrifuged at 160,000g for 2 hours. ATPase was measured by the method of Wakabayashi and Gunge (163).

Fig 5.10

Derepression of D22 A19

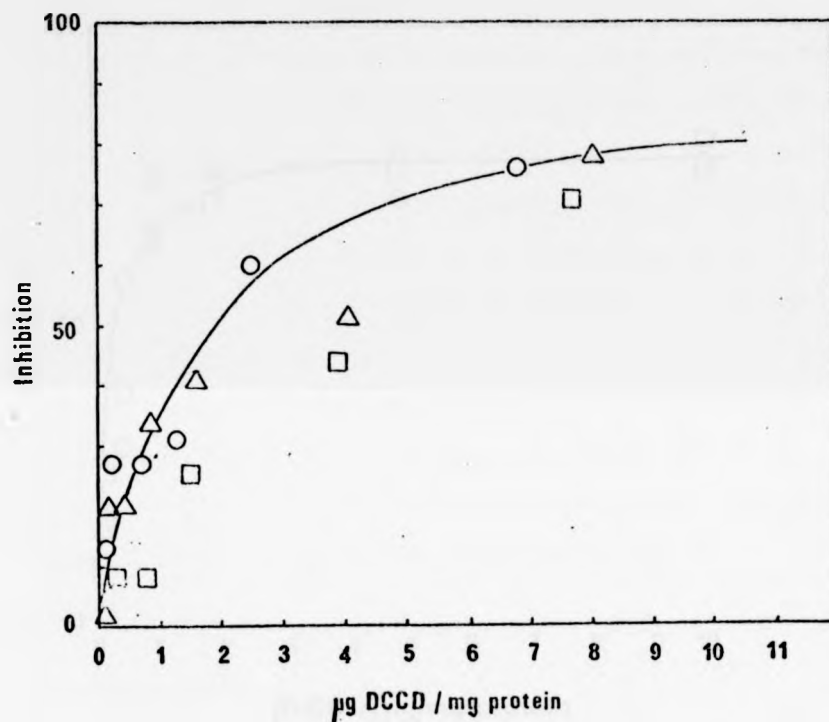


FIGURE 5.10.

Derepression of yeast in the presence of chloramphenicol and cycloheximide was carried out by the method outlined in the text. DCCD was preincubated with submitochondrial particles for 18 hours at 0°C . ATPase assays were performed as described by Wakabayashi and Gunge (163).

□—□ 1/2 hours, △—△ 3 hours, ○—○ 6 hours.

Fig 5.11

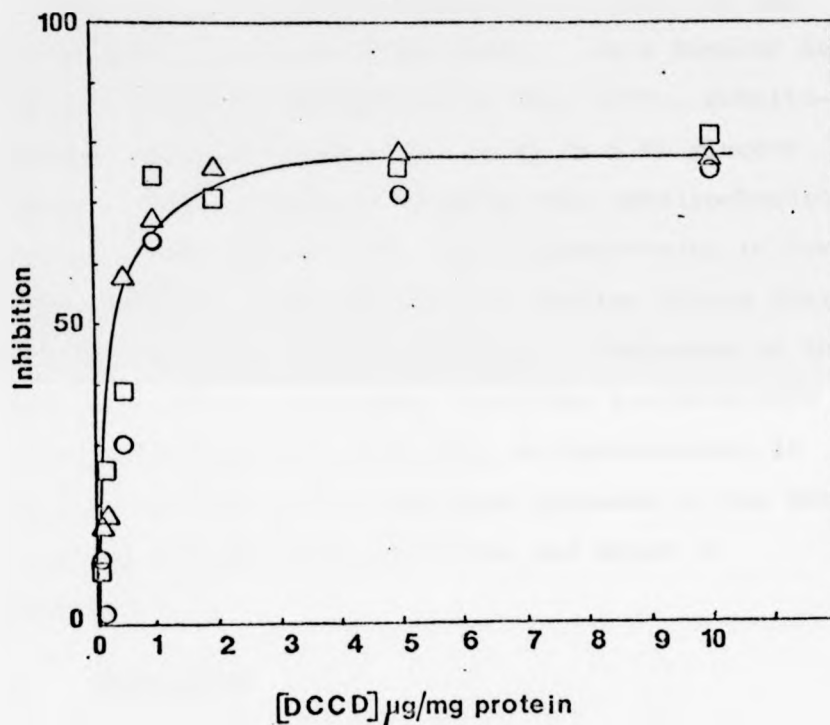
SENSITIVITY OF Mg^{2+} ATPase TO DCCD (D22 A21)

FIGURE 5.11.

Derepression of yeast in the presence of chloramphenicol and cycloheximide was carried out by the method outlined in the text. DCCD was preincubated with submitochondrial particles for 18 hours at 0°C . ATPase assays were performed as described by Wakabayashi and Gunge (163).

○—○ 0 hours, □—□ 1 hour, △—△ 3 hours.

(Fig. 5.12.), although the lowered total inhibitable ATPase for repressed mitochondrial membrane is not as marked.

Binding of ^{14}C DCCD is markedly affected by the derepression of glucose grown yeast. In a similar derepression experiment illustrated in Fig. 5.13., submitochondrial particles from yeast grown on 5.4% glucose display a higher degree of binding than submitochondrial particles from glucose grown cells derepressing in low (0.8%) glucose. This decrease in binding occurs during the first half hour of derepression. Inclusion of the cytoplasmic protein synthesis inhibitor cycloheximide abolishes the decrease in binding on derepression in 0.8% glucose (Fig. 5.14.) and some increase in the degree of binding is found over the first six hours of derepression.

5.4. Discussion

DCCD is an effective inhibitor of mitochondrial ATPase in yeast, as found by Robertson et al. in beef heart (139). The inhibition of beef heart mitochondrial ATPase may be correlated with a covalent binding of ^{14}C DCCD to a proteolipid component of the mitochondrial membrane (78), which may be a subunit of mitochondrial ATPase (77). No such covalent binding has been demonstrated in yeast mitochondria (152, 190), and Broughall (152) has suggested that the covalently bound DCCD is removed by a membrane nucleophile. This would tend to be supported by the decrease in sensitivity found on addition of the external

Fig 5.12

Derepression of D22

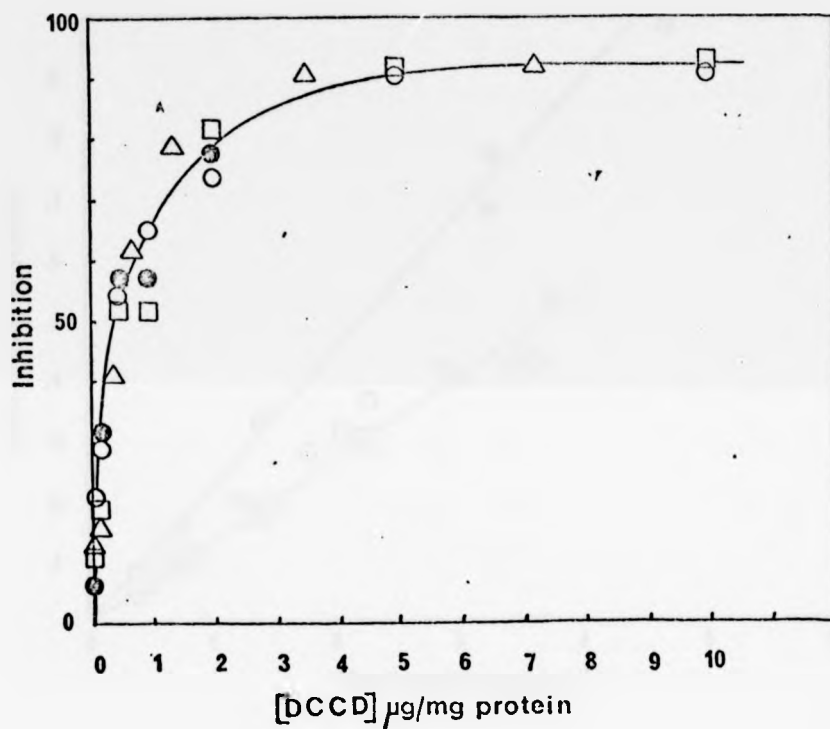


FIGURE 5.12.

Derepression of D22 in the absence of protein synthesis inhibitors was carried out as described in the text. DCCD was incubated with submitochondrial particles for 18 hours at 0°C. ATPase assays were performed as described by Wakabayashi and Gunge (163).

●—● 0 hours, □—□ 3 hours,
○—○ 6 hours, △—△ 12 hours.

Fig 5.13

DCCD BINDING DURING DEREPRESSION

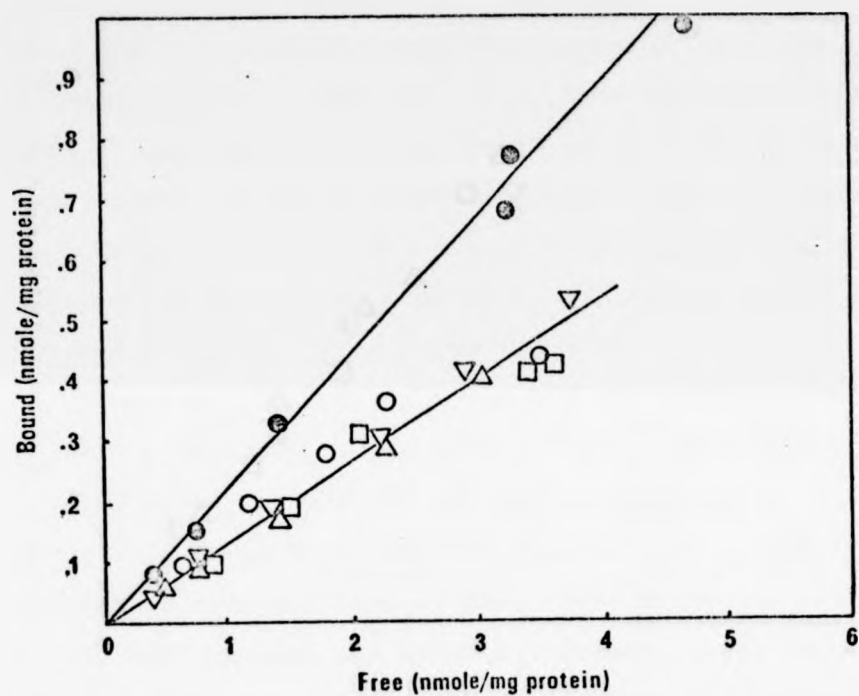


FIGURE 5.13.

S. cerevisiae D22 was grown in 5.4% glucose for 12 hours, harvested and inoculated into 0.8% glucose medium. Aliquots were harvested at 0, $\frac{1}{2}$, 1, 3, 18 hours. Submitochondrial particles were prepared as described by Watson *et al.* (186). Binding of ^{14}C DCCD was performed as described in Methods.

●—● 0 hours, ▽—▽ $\frac{1}{2}$ hour, △—△ 1 hour,
 □—□ 3 hours, ○—○ 18 hours.

Fig 5.14

DCCD BINDING DURING DEREPRESSION IN CYCLOHEXIMIDE

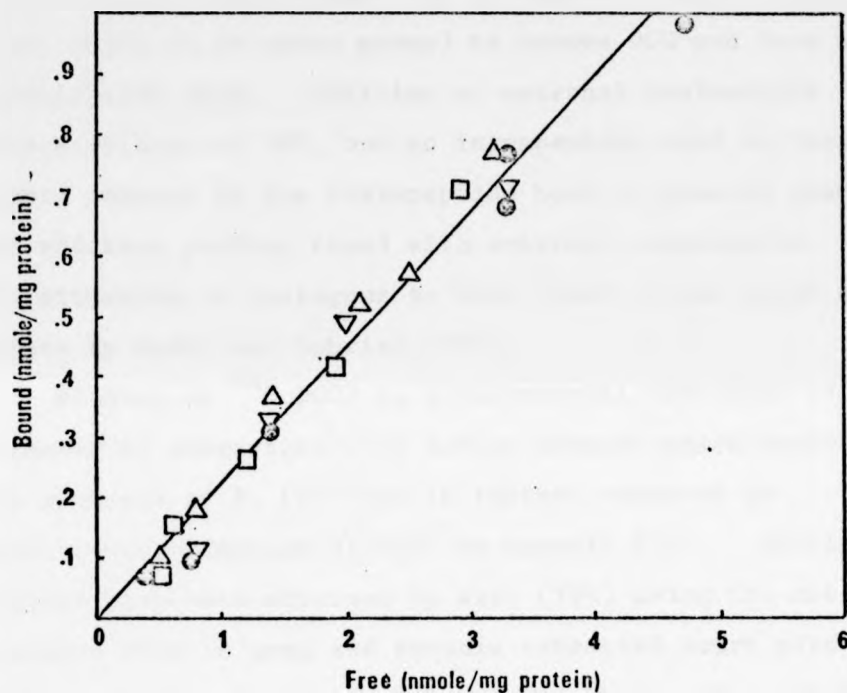


FIGURE 5.14.

S. cerevisiae D22 was grown in 5.4% glucose for 12 hours, harvested and inoculated into 0.8% glucose medium containing $10^{-5}M$ cycloheximide. Aliquots were harvested at 0, $\frac{1}{2}$, 1, 3, 18 hours. Submitochondrial particles were prepared as described by Watson *et al.* (186). Binding of ^{14}C DCCD was performed as described in Methods.

●—● 0 hour, ▽—▽ $\frac{1}{2}$ hour, △—△ 1 hour,
 □—□ 3 hours, ○—○ 18 hours.

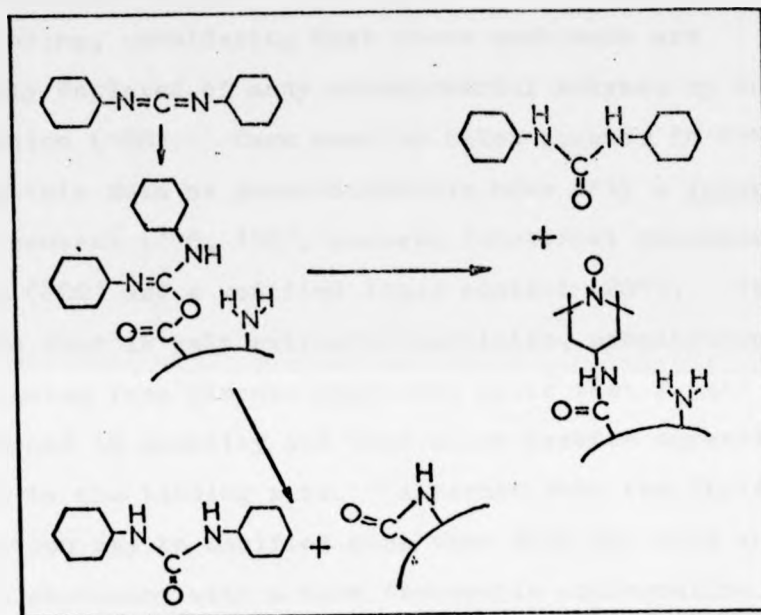
134

nucleophile 4-amino-2,2,6,6-tetramethylpiperid-1-yloxy. A proposed mechanism for the decrease in sensitivity is shown in Fig. 5.15. In the case of DCCD inhibition, the DCCD adduct is attacked by a neighbouring nucleophile (here shown as an amino group) to remove DCU and form an intrapeptide bond. Addition of external nucleophile causes release of DCU, but no intrapeptide bond is formed. Strain induced by the intrapeptide bond is greater than in the addition product found with external nucleophile. The situation is analogous to that found in red blood cell ATPase by Godin and Schrier (191).

Binding of ^{14}C DCCD to mitochondrial membranes is enhanced by extraction with sodium bromide which depletes the membrane of F_1 (70) and is further enhanced by subsequent extraction of OSCP by ammonia (70). Similar results have been obtained by Azzi (195) using the nitroxide analogue NCCD in urea and ammonia extracted heart mitochondrial particles. Azzi has found that only particles which are depleted of both F_1 and OSCP have altered binding characteristics and on the basis of ascorbate reduction of the covalently bound probe, ascribes the altered binding to increased accessibility of the binding site to the aqueous environment (195). This may be the reason for the altered binding in depleted yeast membranes, but these are fundamental differences between the situation found in beef heart and yeast mitochondria. Increased binding is found in submitochondrial particles depleted of F_1 only in yeast, and preliminary results with NCCD in yeast mitochondria (196, 197) suggest that the lipid environment

Fig 5.15

PROPOSED MECHANISM FOR RELIEF OF DCCD
INHIBITION OF Mg^{2+} -ATPase



of the yeast NCCD binding site is more involved in binding than that in beef heart.

It is perhaps not surprising that promitochondria from anaerobically grown yeast possess an enhanced ^{14}C DCCD binding, considering that these membranes are naturally depleted of many mitochondrial enzymes by dedifferentiation (188). Care must be taken however in interpreting this data as promitochondria have only a lowered ATPase content (198, 199), possess functional phosphorylation (200) and a modified lipid content (201). It is possible that in salt extracted particles, promitochondria, or particles from glucose repressed cells that F_1 and OSCP are reduced in quantity and thus allow greater accessibility of DCCD to the binding site. Alternatively the lipid composition may be modified such that DCCD may bind to a protein component with a more favourable conformation. The latter suggestion may be connected with availability of membrane nucleophile (152). The binding data suggests that DCCD binding in F_1 depleted SMP and promitochondrial particles are due to a similar change in the binding site as binding parameters are the same in both cases (Fig. 5.16.).

The identity of this binding site with subunit 9 of the OS-ATPase is shown by (a) its extractibility into chloroform-methanol, indicating the proteolipid nature of the binding site: TLC of extracted binding site shows absence of either free DCU or DCCD. (b) the presence of the binding site in the OS-ATPase complex as shown by precipitation with antisera to OS-ATPase. (c) Co-migration

Fig 5.16

DCCD BINDING TO YEAST SUBMITOCHONDRIAL PARTICLES

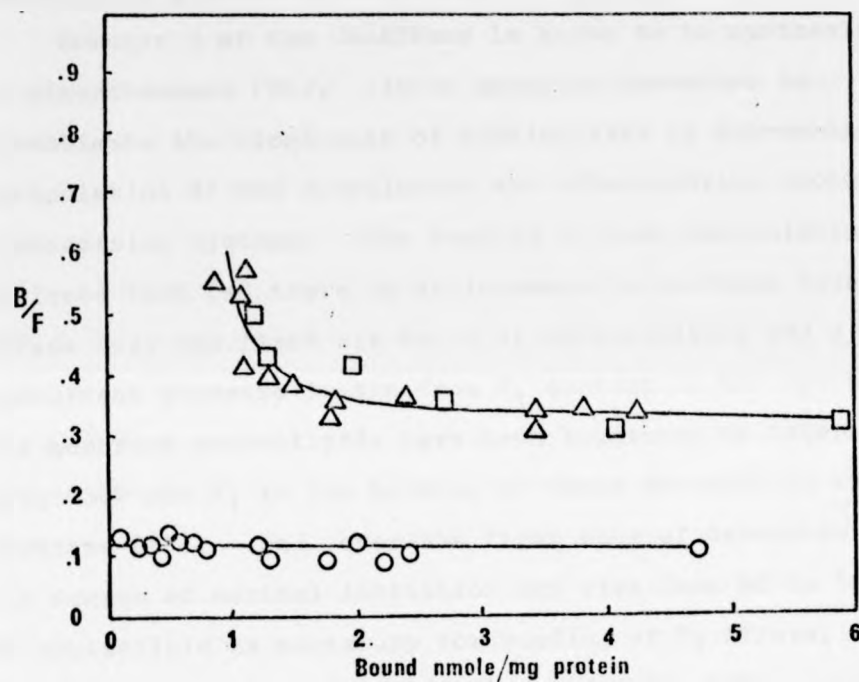


FIGURE 5.16.

Collected data from Figures 5.3., 5.4.

- $\triangle-\triangle$ Y_{ETP} (NaBr, NH_4OH)
 $\square-\square$ promitochondrial particles
 $\circ-\circ$ mitochondrial particles.

with subunit 9 in SDS-polyacrylamide gels. This agrees with the findings of Cattell et al. (78) and Stekhoven (77) that the binding site of DCCD in beef heart mitochondria is a proteolipid subunit of OS-ATPase.

Subunit 9 of the OS-ATPase is known to be synthesised on mitoribosomes (76). It is possible therefore to investigate the biogenesis of binding site by appropriate manipulation of the cytoplasmic and mitochondrial protein synthesising systems. The results of such manipulation indicate that (a) there is an increase in membrane bound ATPase over the first six hours of derepression, and a concurrent decrease in the free F_1 content of the cytoplasm. The membrane proteolipids have been suggested to interact with OSCP and F_1 in the binding of these proteins to the membrane (31). (b) over the first hour of derepression, the degree of maximal inhibition may rise from 80 to 100%. If proteolipid is necessary for binding of F_1 ATPase, and also confers oligomycin and DCCD sensitivity (31), it may be that although an ATPase complex is formed, the complex is not functional until the correct conformation, dictated by the lipid environment, is obtained. The rapid change in the maximal inhibition (0.5 to 1 hour) does not correlate with synthesis of ATPase protein which takes place over the first 6 hours of derepression (199).

(c) The I_{50} values for DCCD inhibition are not greatly different. Under the experimental conditions used, this can be taken to mean that there is little DCCD binding site which is not attached to OSCP and F_1 , the inclusion of chloramphenicol building up an excess of these

proteins. Although more DCCD binding site is synthesised in the presence of cycloheximide, this is always bound by the excess of OSCP and F_1 , and the other mitochondrial proteins increase at a similar rate, giving no great change in the I_{50} values for ATPase. (d) ^{14}C DCCD binding during derepression decreases over the first half-hour, correlating well with the proposed conformational change in (b). That this conformational change is produced in some way by a product of the cytoplasmic protein synthesising system is shown by an increase rather than a decrease in ^{14}C DCCD binding found when derepression takes place in the presence of cycloheximide. The cytoplasmic pool of F_1 is not present in cells grown in cycloheximide (91). It is possible that F_1 and OSCP binding promotes a conformational change in the ATPase complex such that DCCD is not bound covalently. Such a system would also be sensitive to changes in the membrane environment, explaining the increased binding in promitochondria and particles from glucose repressed yeast. That the DCCD sensitivity of ATPase is sensitive to its lipid environment has been shown by solvent extraction in Chapter 3. A lipid involvement in the binding of NCCD to promitochondria has been shown in parallel experiments to those reported here (196, 197).

CHAPTER C

Isolation and characterisation of subunit 9 of the OS-ATPase from *S. cerevisiae* D22

6.1. Introduction

The oligomycin sensitive ATPase consists of (a) the oligomycin insensitive ATPase (F_1) (b) OSCP, a basic coupling factor (c) a membrane factor which confers inhibitor sensitivity on F_1 ATPase. The membrane factor comprises four protein subunits of the ATPase complex which are mitochondrially synthesised (75), and one of these, subunit 9 is probably the site of action of DCCD. The purification and characterisation of the membrane factors has been hampered by their highly hydrophobic and insoluble nature. Several studies have been reported in which subunits of the ATPase complex have been extracted and partially purified (78, , 76) from beef heart, rat liver and yeast, using mixtures of organic solvents. Sierra and Tzagoloff (76) have extensively purified the yeast protein and found that it has a polarity of 0.23 according to the formulation of Cavaldi and Vanderkooi (202), the most hydrophobic protein isolated thus far. The extreme insolubility of this proteolipid has made characterisation of the subunit very difficult and has necessitated the development of novel techniques.

6.2. Methods and Materials

Purification of subunit 9

Mitochondrial particles were prepared from aerobically

grown yeast as previously described (186). Submitochondrial particles were washed with 10 volumes of methanol and the pellet extracted with 20 volumes of Chloroform/Methanol (2/1 v/v) for 18 hours according to Cattell et al. (78). The Chloroform methanol extract was freed of membranes by filtration through a glass sinter. The chloroform-methanol extract was washed with 1/5 volume of distilled water and the organic (lower) phase reduced to 1/5 volume on a rotary evaporator. The washed extract was precipitated with cold (-20°C) diethyl ether (5 vol.) and washed twice with a further 5 volumes of diethyl ether. The precipitate was dissolved in Chloroform/Methanol (2:1 v/v) and separated on Silica gel PLC plates (Merck) in Chloroform/Methanol/Water (65:25:4) containing 20mM HCl. Band 2 was scraped from the PLC plate, extracted three times with Chloroform/Methanol (2:1 v/v) containing 20mM HCl. The pooled, concentrated extracts were applied to a second Silica gel plate and developed in Chloroform/Methanol /17% NH_3 (2:2:1 v/v). Band 4 was eluted into acidic chloroform methanol and precipitated with 5 vols. cold diethyl ether.

Tryptic digestion of proteolipid

Subunit 9 was covalently coupled to glass beads by a modification of the method of Mason and Weetall (203), p-nitrobenzylsulphonyl chloride replacing p-nitrobenzoyl chloride. Tryptic digestion of the bound proteolipid was performed as described by Bennett (204). Peptides were labelled with dansyl chloride (205), and separated on polyamide thin layers by a modification of the method

of Woods and Wang (206). Solvent in the first dimension was 1.5% formic acid, in the second dimension, n-heptane/n-butanol/glacial acetic acid (3:3:1).

Partial Acid Hydrolysis

Cleavage of the purified proteolipid at aspartic acid was performed as described by Tsung (207) in 0.03N hydrochloric acid. Peptides were labelled with dansyl chloride (205) and separated by the method of Woods and Wang (206).

N-terminal determination

Identification of the N-terminal amino acid was performed as described by Gray (208). Dansylated proteolipid was hydrolysed in 6.7N hydrochloric acid at 100°C and the N-terminal amino acid identified by the method of Hartley (209).

Polyacrylamide gel electrophoresis

Electrophoresis of the proteolipid in SDS containing solvent was as described by Weber and Osbourne (143). Gels were stained in 1% amido black and destained in 7% acetic acid. Gels containing radioactivity were sliced using a stacked razor blade assembly (210), and 1mm slices dissolved in 6% hydrogen peroxide prior to scintillation counting in Triton X-100/toluene/butyl PBD (144).

6.3. Results

Purification of proteolipid

The purification of a protein which has no enzymic

properties and no coupling factor activity presents certain problems in the determination of purification, yield and purity. The presence of the proteolipid has been monitored by the incorporation of ^3H leucine into the products of mitochondrial protein synthesis. Subunit 9 is known to be synthesised on mitoribosomes (76). A typical preparation from ^3H labelled submitochondrial particles is shown in Table 6.1. It can be seen that large amounts of other material are still present as contaminants of the proteolipid spot in the first TLC run (Figs. 6.1., 6.2.). The extraction method removes more protein from SMP than that of Sierra and Tzagoloff (76) and the final yield is about eight times higher. Although washing steps are included in the preparative method, the presence of contaminants even after the first chromatographic separation suggests that the extraction procedure is not as specific for subunit 9 as that of Sierra and Tzagoloff (76). Attempts at separation of the yeast proteolipid on Sephadex LH20 columns developed in varying concentrations of chloroform-methanol gave a number of protein peaks, probably due to the aggregation of protein on the column, or differences in solubility of more or less denatured protein. It was found that the precipitated protein was very difficult to redissolve and that the further along the preparative procedure, the greater the insolubility. This behaviour is found in both rat liver and beef heart mitochondrial proteolipids, and is responsible for major losses of material.

The homogeneity of the proteolipid may be assessed

TABLE 6.1. Purification of subunit 9

Total protein (mg)	cpm ($\times 10^{-3}$)	Stage
250	340	SMP
10.1	56.2	CHCl ₃ /MeOH extract
7.6	52.1	Washed extract
5.5	48.0	Ether precipitate
3.0	27.2	TLC spot 1
2.1	23.6	TLC spot 2

Purification of subunit 9 proteolipid was carried out as described in Methods. TLC spot 1 is band 2 from the first TLC run, TLC spot 2 is band 4 from the second TLC run.

Fig 6.1

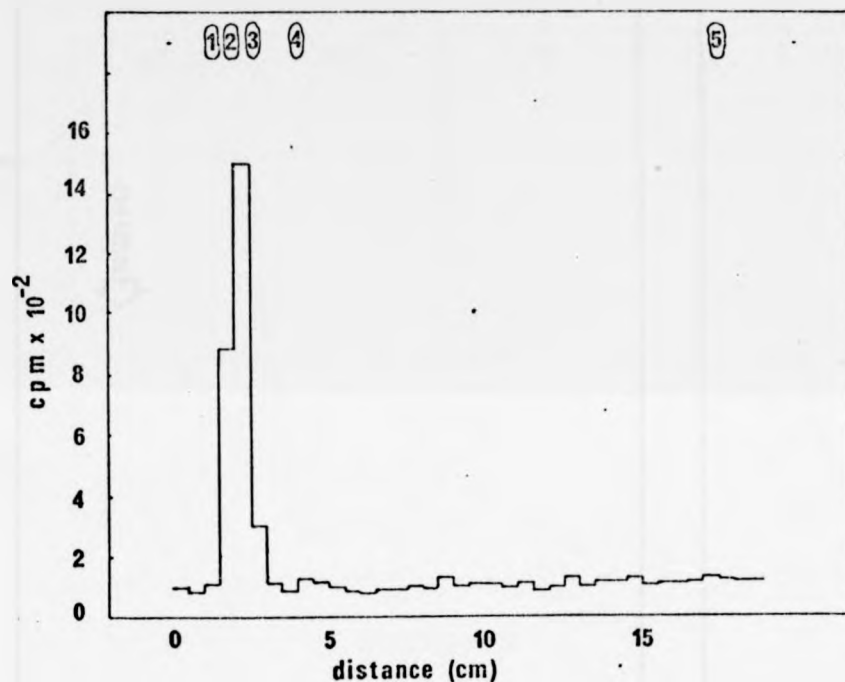
TLC OF YEAST ^3H SUBUNIT 9

FIGURE 6.1.

^3H leucine was incorporated into yeast submitochondrial particles in the presence of cycloheximide as described by Tzagoloff (70). Subunit 9 was prepared as outlined in Methods. Crude ether was run on a preparative layer (5mm) Silica gel plate in Chloroform/Methanol/Water/6N HCl (130:50:7.2:0.72). Spots were visualised with iodine vapour.

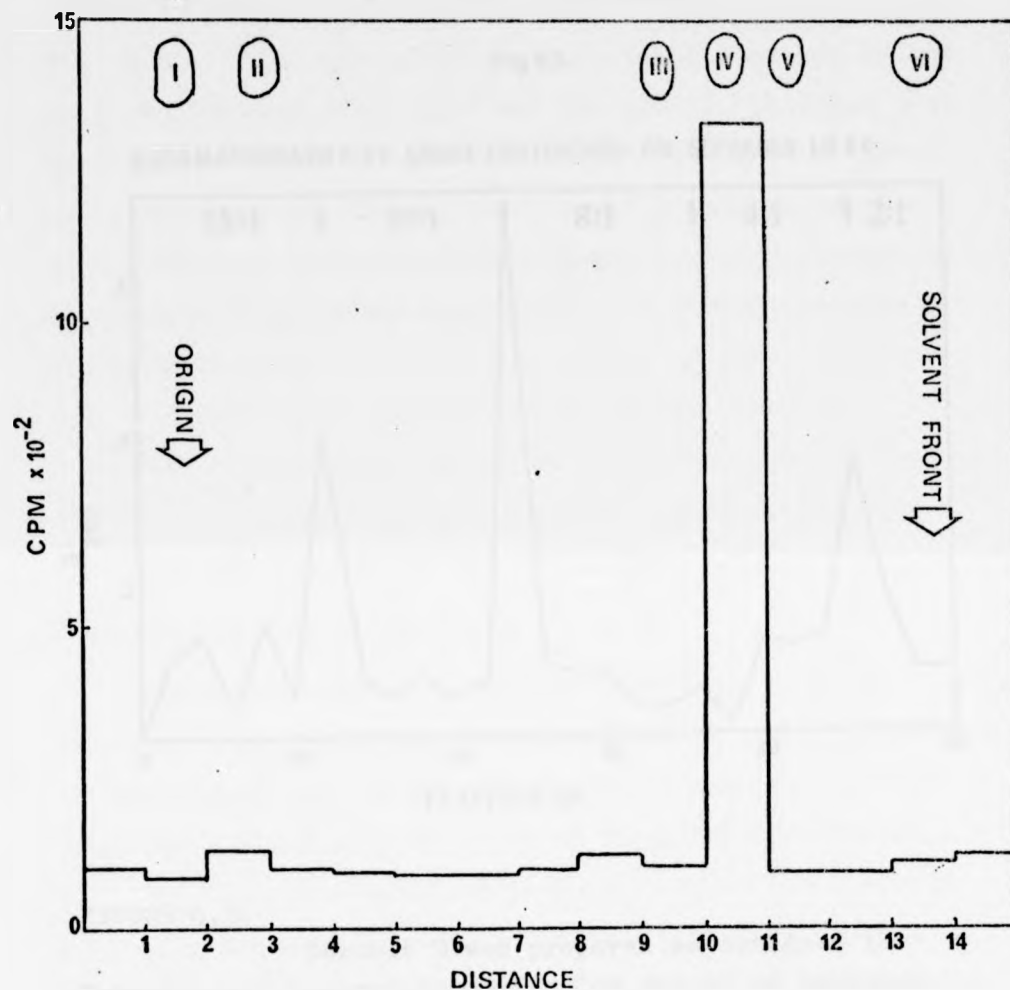
TLC OF YEAST ^3H -SUBUNIT 9

FIGURE 6.2.

^3H leucine was incorporated into yeast sub-mitochondrial particles in the presence of cycloheximide as described by Tzagoloff (70). Subunit 9 was prepared as outlined in Methods. Crude ether precipitate was run on a preparative layer (5mm) Silica gel plate in Chloroform/Methanol/17% NH_3 (2:2:1 v/v). Spots were visualised with iodine vapour.

Fig 6.3

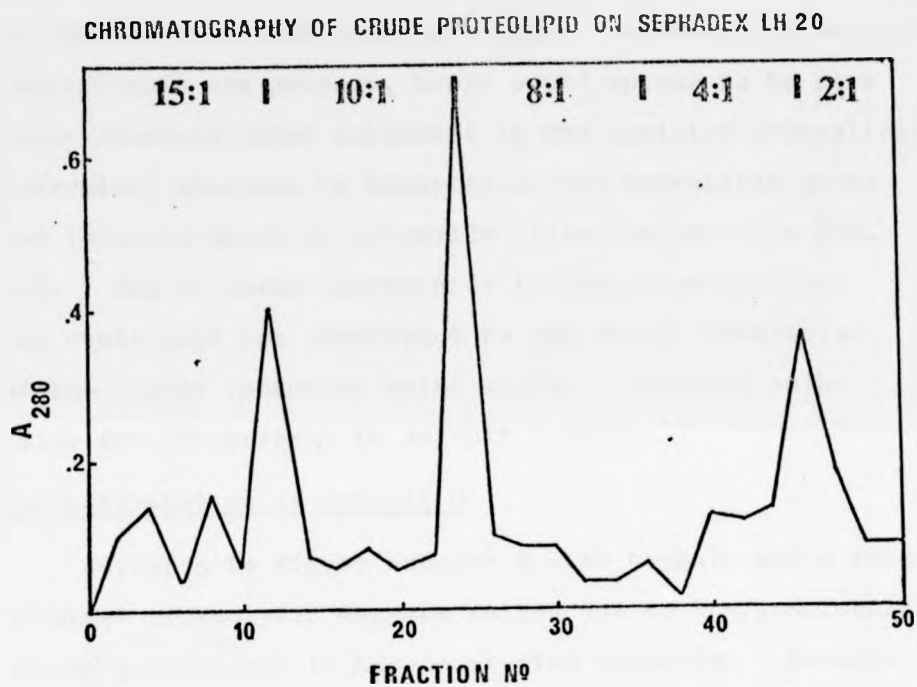


FIGURE 6.3.

Subunit 9 was prepared as outlined in Methods, and layered on a 20 x 1cm column of Sephadex LH20 in Chloroform. Protein was eluted by sequential addition of 50ml of Chloroform/Methanol 15:1 (v/v), 10:1 (v/v), 8:1 (v/v), 4:1 (v/v), and 2:1 (v/v).

by polyacrylamide gel electrophoresis as illustrated in Fig. 6.4. ^{14}C -DCCD labelled proteolipid migrates as one peak on SDS containing gels and one protein staining band is found to coincide with this peak. Although no protein contaminants are present, there would appear to be some other strongly bound component in the purified proteolipid. N-terminal analysis by dansylation and hydrolysis gives two labelled spots on polyamide plates as shown in Fig. 6.5. One of these corresponds to dansyl-methionine, the other does not correspond to any dansyl derivative of the twenty commonest amino acids. Uncommon amino acids are not present in subunit 9 (76).

Characterisation of subunit 9

Attempts to digest subunit 9 with trypsin and a range of other proteolytic enzymes failed due to the insolubility of the proteolipid in non-denaturing solvents. Subunit 9 bound to a glass support could be digested with trypsin, but this technique required large amounts of proteolipid. A tryptic digest (fingerprint) of subunit 9 from the parental strain D22 is shown in Fig. 6.6. The theoretical number of spots from a normal tryptic digestion is 4: in this case the proteolipid is anchored to the glass support by at least one $-\text{NH}_2$ side chain, and consequently the maximum theoretical number of tryptic peptides is 7. Few peptides are found in the digest in detectable quantities and it is possible that minor components are present at very low levels.

No differences were found between DCCD mutant

SDS-GEL ELECTROPHORESIS OF SUBUNIT 9

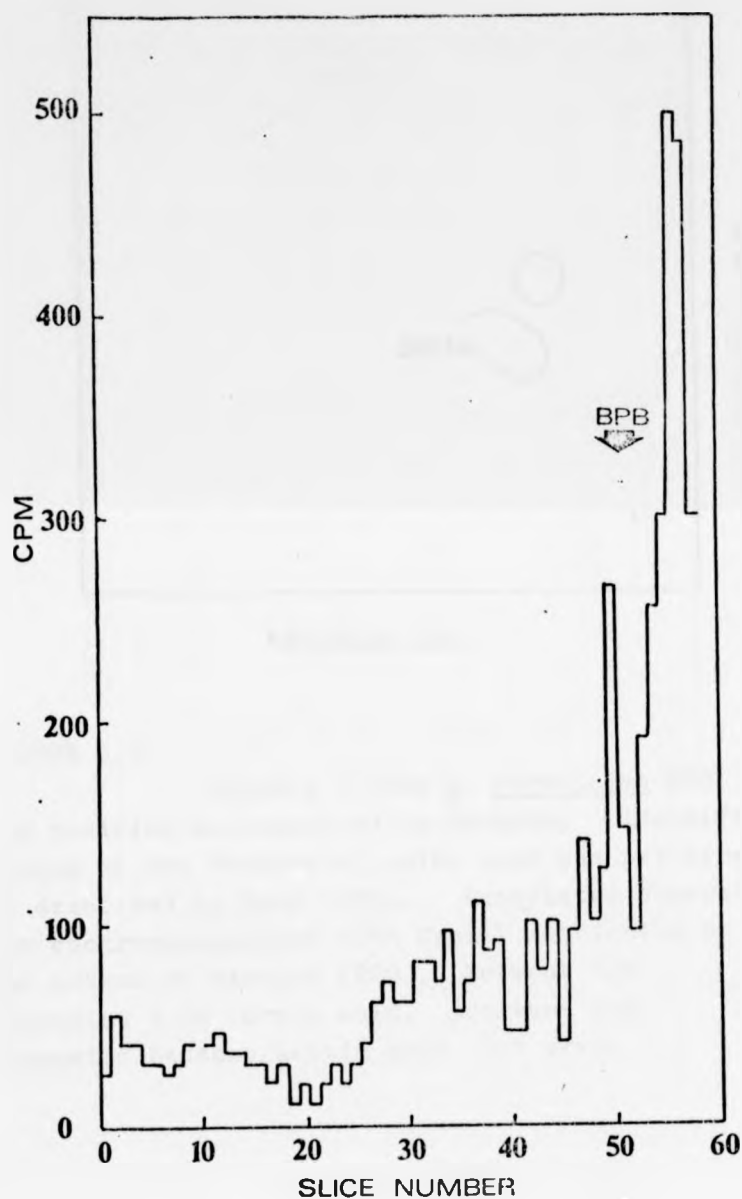


FIGURE 6.4.

Subunit 9 was prepared as outlined in Methods. ^{14}C DCCD was bound to subunit 9 in Chloroform/Methanol 2:1 (v/v) and precipitated with cold diethyl ether. The precipitate was dissolved in 3% SDS, 5% mercaptoethanol, phosphate buffer 100mM pH 8.5. Electrophoresis was performed as described by Weber and Osbourne (143).

Fig 6.5

N-TERMINAL AMINO ACID DETERMINATION : SUBUNIT 9

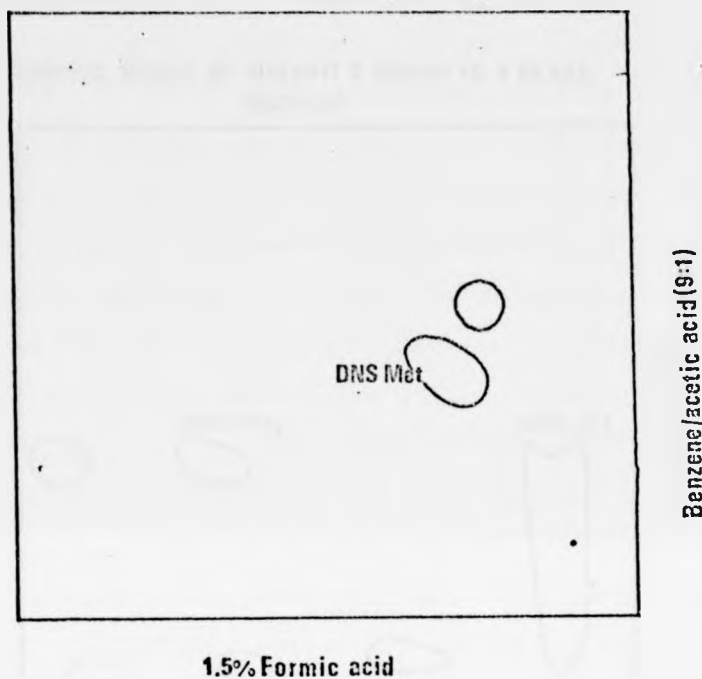


FIGURE 6.5.

Subunit 9 from *S. cerevisiae* D22 was purified as described in Methods. Identification of the N-terminal amino acid was performed as described by Gray (208). Dansylated N-terminal was cochromatographed with dansyl methionine by the method of Hartley (209). Solvent 1st dimension 1.5% formic acid. Solvent 2nd dimension Benzene/acetic acid (9:1 v/v).

Fig 6.6

TRYPTIC DIGEST OF SUBUNIT 9 BOUND TO A GLASS
SUPPORT

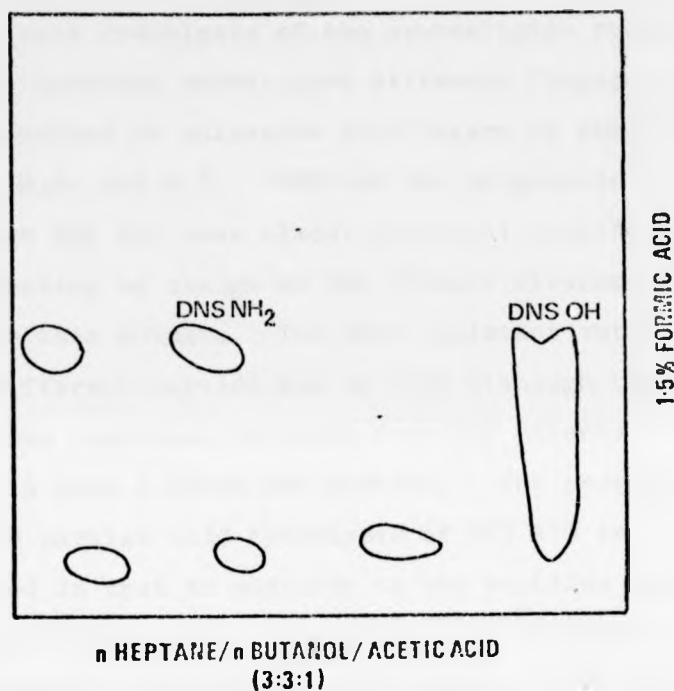


FIGURE 6.6.

Subunit 9 from *S. cerevisiae* was prepared as described in the text. Subunit 9 was covalently coupled to glass beads by the method of Mason and Weetall (203). Tryptic digestion was as described by Bennett (204). Peptides were separated on polyamide plates. Solvent 1st dimension 1.5% formic acid, solvent 2nd dimension n-Heptane/n-Butanol/acetic acid (3:3:1 v/v).

preparations and proteolipid preparations from the parental strain during purification. Subunit 9 from a number of mutants had the same R_f values as shown in Table 6.2. Partial acid hydrolysis of the proteolipids from mutants and the parental strain gave different fingerprints when separated on polyamide thin layers as shown in Figs. 6.7., 6.8. and 6.9. D22 and the oligomycin resistant mutant D22 A21 have almost identical peptide patterns, indicating no change in the primary structure of subunit 9 in this mutant. The DCCD resistant mutant D22 61 has a different peptide map in that although there are five peptides identical to those from the parental strain, there is also a sixth new peptide. The peptide map produced by partial acid hydrolysis of D22 A19 is markedly altered in that in addition to the peptides found for the parental strain, two new peptides are produced, and the positions of the peptides are altered. Unfortunately, some core protein is not digested by partial acid hydrolysis under the very mild conditions used, but hydrolyses in higher concentrations of acid were unsatisfactory due to poor reproducibility.

6.4. Discussion

A major problem in the interpretation of binding data relating to multisubunit enzymes such as the oligomycin sensitive ATPase complex is that a lesion in one of the subunits may alter the conformation of the complex such that binding of ligand to a second subunit is diminished. For this reason, it has previously been difficult to relate

TABLE 6.2. Relative mobilities of subunit 9 from
varying sources

R_f	Source
0.72	<u>S. cerevisiae</u> D22
0.72	<u>S. cerevisiae</u> D22 A15
0.70	<u>S. cerevisiae</u> D22 A19
0.72	<u>S. cerevisiae</u> D22 A21
0.73	<u>S. cerevisiae</u> D22 61
0.72	Beef Heart mitochondria

Crude proteolipids extracted from submitochondrial particles with chloroform/methanol (2:1 v/v) and precipitated with diethyl ether as described by Cattell (78) were chromatographed on Silica Gel plates in chloroform/methanol/17% NH_3 (2:1:1 v/v/v). Spots were visualised with iodine vapour.

Fig 6.7

Partial acid hydrolysis of subunit 9 D22 A21

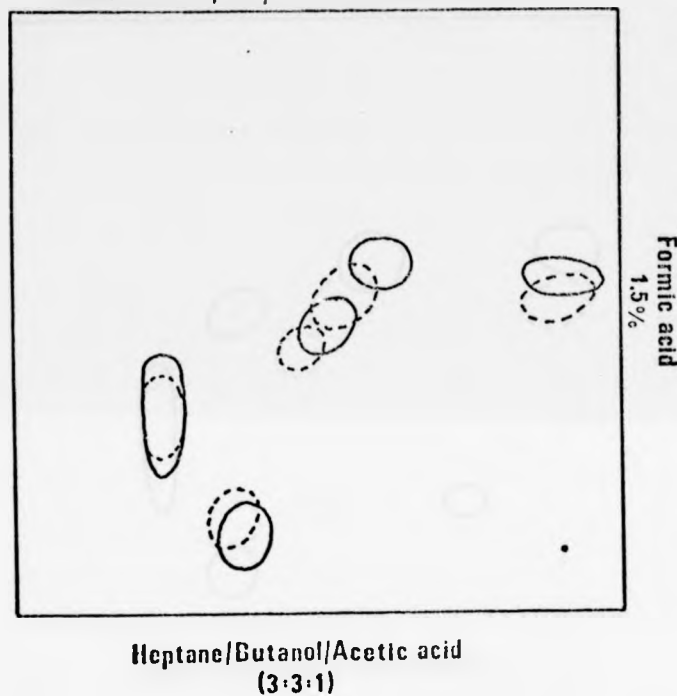


FIGURE 6.7.

Cleavage of purified subunit 9 at aspartic acid was performed as described by Tsung (207). Dansylated peptides were separated on polyamide plates. Solvent 1st dimension 1.5% acetic acid, solvent 2nd dimension Heptane/Butanol/acetic acid (3:3:1 v/v). Dashed lines are peptides from D22 parental. Solid lines refer to peptides from the mutant strains.

Fig 6.8

Partial acid hydrolysis of subunit 9 D22 61

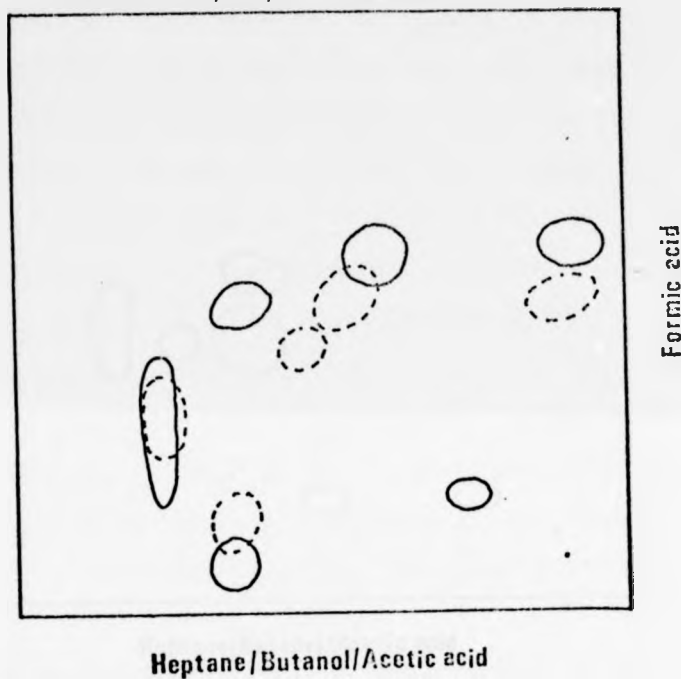


FIGURE 6.8.

Cleavage of purified subunit 9 at aspartic acid was performed as described by Tsung (207). Dansylated peptides were separated on polyamide plates. Solvent 1st dimension 1.5% acetic acid, solvent 2nd dimension Heptane/Butanol/acetic acid (3:3:1 v/v).

Fig 6.9

Partial acid hydrolysis of subunit 9 D22 A19

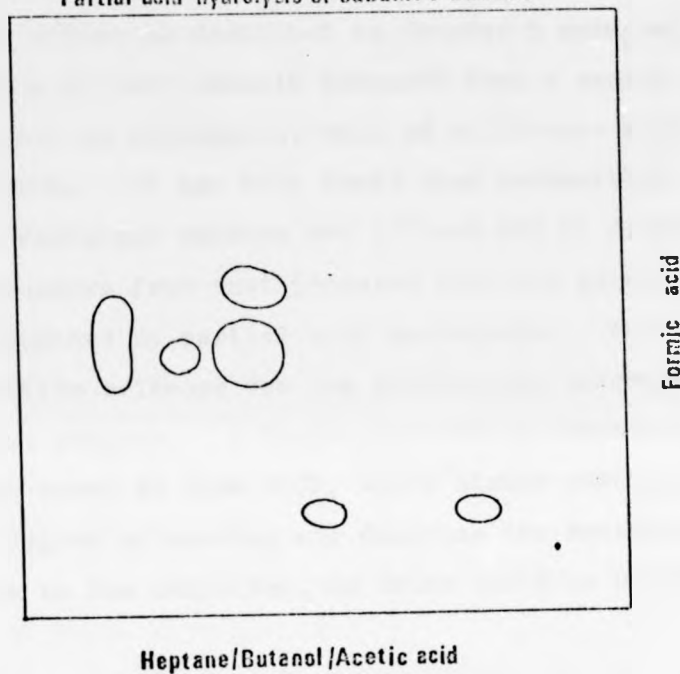


FIGURE 6.9.

Cleavage of purified subunit 9 at aspartic acid was performed as described by Tsung (207). Dansylated peptides were separated on polyamide plates. Solvent 1st dimension 1.5% acetic acid, solvent 2nd dimension Heptane/Butanol/acetic acid (3:3:1 v/v).

the genetic evidence obtained with inhibitor resistant mutants, to specific binding sites in the OS-ATPase complex. The ~~finding~~ that DCCD binds to a specific subunit of the ATPase as described in Chapter 5 prompted an investigation of this subunit prepared from a series of mutants resistant to oligomycin, some of which were also resistant to DCCD. It has been found that proteolipid from the DCCD resistant mutants D22 A19 and D22 61 differs in primary structure from that prepared from the parental strain as determined by partial acid hydrolysis. This is good supportive evidence for the conclusions reached in the previous chapter. A lesion induced in subunit 9, which has been shown to bind DCCD, would almost certainly decrease the degree of binding and decrease the sensitivity of the complex to the inhibitor, as found for both D22 A19 and D22 61.

The OL111 mutants D22 A19 and D22 61 are also resistant to oligomycin, as is the OL1 mutant D22 A21. D22 A21 subunit 9 does not differ from that of the parental strain. Without access to labelled oligomycin, one cannot rule out the possibility of subunit 9 being the binding site of oligomycin: the OL111 mutants are resistant to oligomycin and the resistance to any inhibitor may arise as a cooperative effect between subunits.

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